

Ans. A1

TYROSINE KINASE RECEPTOR EphA3 ANTIGENIC PEPTIDESField of the Invention

This invention relates to fragments of the tumor associated gene product EphA3 which  
5 bind to and are presented to T lymphocytes by HLA molecules. The peptides, nucleic acid  
molecules which code for such peptides, as well as related antibodies and T lymphocytes, are  
useful, *inter alia*, in diagnostic and therapeutic contexts.

Background of the Invention

10 The process by which the mammalian immune system recognizes and reacts to foreign  
or alien materials is complex. An important facet of the system is the T cell response, which  
in part comprises mature T lymphocytes which are positive for either CD4 or CD8 cell surface  
proteins. T cells can recognize and interact with other cells via cell surface complexes on the  
other cells of peptides and molecules referred to as human leukocyte antigens ("HLAs") or  
15 major histocompatibility complexes ("MHCs"). The peptides are derived from larger  
molecules which are processed by the cells which also present the HLA/MHC molecule. See  
Male et al., Advanced Immunology (J.P. Lipincott Company, 1987), especially chapters 6-10.  
The interaction of T cells and complexes of HLA/peptide is restricted, requiring a specific T  
cell for a specific complex of an HLA molecule and a peptide. If a specific T cell is not  
20 present, there is no T cell response even if its partner complex is present. Similarly, there is  
no response if the specific complex is absent, but the T cell is present. The mechanisms  
described above are involved in the immune system's response to foreign materials, in  
autoimmune pathologies, and in responses to cellular abnormalities.

The T cell response to foreign antigens includes both cytolytic T lymphocytes and  
25 helper T lymphocytes. CD8<sup>+</sup> cytotoxic or cytolytic T cells (CTLs) are T cells which, when  
activated, lyse cells that present the appropriate antigen presented by HLA class I molecules.  
CD4<sup>+</sup> T helper cells are T cells which secrete cytokines to stimulate macrophages and antigen-  
producing B cells which present the appropriate antigen by HLA class II molecules on their  
surface.

30 The mechanism by which T cells recognize alien materials also has been implicated in  
cancer. A number of cytolytic T lymphocyte (CTL) clones directed against autologous  
melanoma have been described. In some instances, the antigens recognized by these clones

have been characterized. In De Plaen et al., *Immunogenetics* 40:360-369 (1994), the "MAGE" family, a family of genes encoding tumor specific antigens, is described. (See also PCT application PCT/US92/04354, published on November 26, 1992.) The expression products of these genes are processed into peptides which, in turn, are expressed on cell surfaces. This can lead to lysis of the tumor cells by specific CTLs. The genes are said to code for "tumor rejection antigen precursors" or "TRAP" molecules, and the peptides derived therefrom are referred to as "tumor rejection antigens" or "TRAs". See Traversari et al., *Immunogenetics* 35: 145 (1992); van der Bruggen et al., *Science* 254: 1643 (1991), for further information on this family of genes. Also, see U.S. Patent No. 5,342,774.

The foregoing references describe isolation and/or characterization of tumor rejection antigens which are presented by HLA class I molecules. These TRAs can induce activation and proliferation of CD8<sup>+</sup> cytotoxic T lymphocytes (CTLs) which recognize tumor cells that express the tumor associated genes (e.g. MAGE genes) which encode the TRAs.

The importance of CD4<sup>+</sup> T lymphocytes (helper T cells) in antitumor immunity has been demonstrated in animal models in which these cells not only serve cooperative and effector functions, but are also critical in maintaining immune memory (reviewed by Topalian, *Curr. Opin. Immunol.* 6:741-745, 1994). Moreover, several studies support the contention that poor tumor-specific immunity is due to inadequate activation of T helper cells.

It has recently been demonstrated that the tyrosinase gene encodes peptides which are presented by HLA class II molecules to stimulate CD4<sup>+</sup> T lymphocytes (Topalian et al., 1994; Yee et al., *J. Immunol.* 157:4079-4086, 1996; Topalian et al., *J. Exp. Med.* 183:1965-1971, 1996).—See also PCT publication number WO97/11669.

As with many cancer associated antigens, tyrosinase is expressed in a limited percentage of tumors and in limited types of tumors. Furthermore, the two identified MHC class II binding tyrosinase peptides are HLA-DRB1\*0401-restricted peptides, recognized only by cells which express the particular HLA molecule.

More recently, HLA class II binding peptides of the MAGE-3 protein were identified which stimulate CD4<sup>+</sup> T lymphocytes (see international application number PCT/US98/18601).

There exist many patients who would not benefit from any therapy which includes helper T cell stimulation via tyrosinase or MAGE-3 peptides, either because the patient's tumor does not express tyrosinase or MAGE-3, or because the patient does not express the

appropriate HLA molecule. Accordingly, there is a need for the identification of additional tumor associated antigens which contain epitopes presented by MHC class II molecules and recognized by CD4<sup>+</sup> lymphocytes.

### Summary of the Invention

It now has been discovered that the EphA3 gene encodes an antigen which contains HLA class II binding peptides. These peptides, when presented by an antigen presenting cell having an HLA class II molecule, effectively induce the activation and proliferation of CD4<sup>+</sup> T lymphocytes.

The invention provides isolated EphA3 peptides which bind HLA molecules, and functional variants of such peptides which retain HLA binding properties, the functional variants comprising one or more amino acid additions, substitutions or deletions to the EphA3 peptide sequence. The invention also provides isolated nucleic acid molecules encoding such peptides, expression vectors containing those nucleic acid molecules, host cells transfected with those nucleic acid molecules, and antibodies to those peptides and complexes of the peptides and HLA antigen presenting molecules. T lymphocytes which recognize complexes of the peptides and HLA antigen presenting molecules are also provided. Kits and vaccine compositions containing the foregoing molecules additionally are provided. The foregoing can be used in the diagnosis or treatment of conditions characterized by the expression of EphA3. As it is known that the members of the Eph family of polypeptides and nucleic acids share significant sequence identity and functional homology (e.g., as tyrosine kinase receptors and precursors), the invention also embraces HLA binding peptides derived from members of the Eph family other than EphA3. Therefore, it is understood that the disclosure contained herein of EphA3 HLA binding peptides, compositions containing such peptides, and methods of identifying and using such peptides applies also to other members of the Eph tyrosine kinase receptor family.

According to one aspect of the invention, an isolated EphA3 HLA class II-binding peptide, comprising a fragment of the amino acid sequence of SEQ ID NO: 3, 5 or 7 which binds an HLA class II molecule, or a functional variant thereof which binds HLA class II molecules comprising one or more amino acid additions, substitutions or deletions, is provided. The isolated peptide in one embodiment is a fragment of the amino acid sequence of SEQ ID NO:3, SEQ ID NO: 5 or SEQ ID NO:7, or a functional variant thereof.

In another aspect of the invention, an isolated peptide comprising the amino acid sequence of SEQ ID NO:53, or a functional variant thereof which binds HLA class II molecules comprising one or more amino acid additions, substitutions or deletions, is provided. The isolated peptide in one embodiment is selected from the group consisting of SEQ ID NO:51, SEQ ID NO:54, SEQ ID NO:62, fragments thereof, and functional variants thereof. In preferred embodiments, the isolated peptide consists of one of the foregoing amino acid sequences. In all compositions embodiments, the isolated peptide is a fragment of EphA3 protein and not the entire EphA3 protein.

In certain embodiments, the isolated peptide comprises an endosomal targeting signal, preferably an endosomal targeting portion of human invariant chain Ii or LAMP-1. In other embodiments of the invention, the isolated HLA class II-binding peptide is non-hydrolyzable. Preferred non-hydrolyzable peptides are selected from the group consisting of peptides comprising D-amino acids, peptides comprising a  $-\text{psi}[\text{CH}_2\text{NH}]$ -reduced amide peptide bond, peptides comprising a  $-\text{psi}[\text{COCH}_2]$ -ketomethylene peptide bond, peptides comprising a  $-\text{psi}[\text{CH}(\text{CN})\text{NH}]$ -(cyanomethylene)amino peptide bond, peptides comprising a  $-\text{psi}[\text{CH}_2\text{CH}(\text{OH})]$ -hydroxyethylene peptide bond, peptides comprising a  $-\text{psi}[\text{CH}_2\text{O}]$ -peptide bond, and peptides comprising a  $-\text{psi}[\text{CH}_2\text{S}]$ -thiomethylene peptide bond.

According to another aspect of the invention, Eph HLA class I binding peptides are provided. The HLA class I binding peptides are fragments of Eph proteins, particularly of EphA3, which bind HLA class I molecules and preferably stimulate  $\text{CD8}^+$  T lymphocytes. Functional variants, non-hydrolyzable peptides, and fusions of HLA class I peptides also are provided.

According to another aspect of the invention, a composition comprising an isolated EphA3 HLA class I-binding peptide and an isolated EphA3 HLA class II-binding peptide is provided.

In certain embodiments, the EphA3 HLA class I-binding peptide and the EphA3 HLA class II-binding peptide are combined as a polytope polypeptide. In other embodiments, the isolated EphA3 HLA class II-binding peptide includes an amino acid sequence selected from the group consisting of SEQ ID NO:51, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:62, fragments thereof, and functional variants thereof. In certain embodiments of the foregoing compositions, the isolated EphA3 HLA class II-binding peptide or polytope includes an endosomal targeting signal. Preferably the endosomal targeting signal includes an endosomal targeting portion of human invariant chain Ii.

According to another aspect of the invention, an isolated nucleic acid encoding any of the foregoing HLA class II-binding peptides or HLA class I binding peptides, or combination thereof is provided. Preferably the nucleic acid comprises or consists of a fragment of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:52 and fragments of SEQ ID NO:52.

According to still another aspect of the invention, expression vectors are provided. The expression vectors comprise any of the foregoing isolated nucleic acids operably linked to a promoter. In preferred embodiments, the nucleic acid comprises or consists of a fragment of SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6, or SEQ ID NO:52, or the minigenes depicted in Fig. 5. In other embodiments, the expression vector further comprises a nucleic acid which encodes an HLA-DRB1\*1101 molecule or another HLA DR11 molecule.

According to yet another aspect of the invention, host cells transfected or transformed with any of the foregoing expression vectors are provided. Host cells which express an HLA-DR11 molecule, and which are transfected or transformed with any of the foregoing expression vectors are also provided.

According to another aspect of the invention, methods for enriching selectively a population of T lymphocytes with CD4<sup>+</sup> T lymphocytes specific for an EphA3 HLA class II-binding peptide are provided. The methods include contacting a source of T lymphocytes with an agent presenting a complex of the EphA3 HLA class II-binding peptide and an HLA class II molecule in an amount sufficient to selectively enrich the population of T lymphocytes with the CD4<sup>+</sup> T lymphocytes. Preferably the source of T lymphocytes is a tissue or a population of cells which contains T lymphocytes. Exemplary sources of T lymphocytes include peripheral blood lymphocytes and lymph nodes. In certain embodiments the source of T lymphocytes is an isolated population of T lymphocytes. In the foregoing embodiments, it is preferred that the sources and/or populations of T lymphocytes are isolated.

In other embodiments, the agent is an antigen presenting cell contacted with an EphA3 protein or an HLA class II binding fragment thereof. In preferred embodiments, the HLA class II molecule is an HLA-DR11 molecule and the EphA3 HLA class II-binding peptide is selected from the group consisting of (i) peptides which consist of a fragment of an amino acid sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:5 and SEQ ID NO:7, (ii) peptides which comprise the amino acid sequence of SEQ ID NO:53 or SEQ ID NO:62, and (iii) functional variants of the peptides of (i) and (ii). In certain embodiments of

the foregoing methods, the isolated EphA3 protein or HLA class II binding peptide thereof includes an endosomal targeting signal. Preferably the endosomal targeting signal includes an endosomal targeting portion of human invariant chain Ii or LAMP-1.

According to a further aspect of the invention, methods for diagnosing a disorder characterized by expression of EphA3 are provided. The methods include contacting a biological sample isolated from a subject with an agent that is specific for the EphA3 HLA class II binding peptide, and determining the interaction between the agent and the EphA3 HLA class II binding peptide as a determination of the disorder. The biological sample in some embodiments is, for example, dendritic cells loaded with a tumor cell lysate. In certain embodiments, the peptide is selected from the group consisting of (i) peptides which consist of a fragment of an amino acid sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:5 and SEQ ID NO:7, (ii) peptides which comprise the amino acid sequence of SEQ ID NO:53 or SEQ ID NO:62, and (iii) functional variants of the peptides of (i) and (ii).

According to another aspect of the invention, methods for diagnosing a disorder characterized by expression of an EphA3 HLA class II-binding peptide which forms a complex with an HLA class II molecule are provided. The methods include contacting a biological sample isolated from a subject with an agent that binds the complex; and determining binding between the complex and the agent as a determination of the disorder. In some embodiments the HLA class II molecule is an HLA-DR11 molecule, such as HLA-DRB1\*1101 and the EphA3 HLA class II-binding peptide is selected from the group consisting of (i) peptides which consist of a fragment of an amino acid sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:5 and SEQ ID NO:7, (ii) peptides which comprise the amino acid sequence of SEQ ID NO:53 or SEQ ID NO:62, and (iii) functional variants of the peptides of (i) and (ii).

Methods for treating a subject having a disorder characterized by expression of EphA3 are provided in another aspect of the invention. The methods include administering to the subject an amount of an EphA3 HLA class II-binding peptide sufficient to ameliorate the disorder. In certain embodiments the EphA3 HLA class II-binding is selected from the group consisting of (i) peptides which consist of a fragment of an amino acid sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:5 and SEQ ID NO:7, (ii) peptides which comprise the amino acid sequence of SEQ ID NO:53 or SEQ ID NO:62, and (iii) functional variants of the peptides of (i) and (ii). In certain embodiments, the EphA3 HLA class II

binding peptide comprises an endosomal targeting signal, preferably an endosomal targeting portion of human invariant chain Ii or LAMP-1.

According to still another aspect of the invention, methods for treating a subject having a disorder characterized by expression of EphA3 are provided. The methods include administering to the subject an amount of an EphA3 HLA class I-binding peptide and an amount of an EphA3 HLA class II-binding peptide sufficient to ameliorate the disorder. In certain embodiments, the EphA3 HLA class II-binding peptide is selected from the group consisting of (i) peptides which consist of a fragment of an amino acid sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:5 and SEQ ID NO:7, (ii) peptides which comprise the amino acid sequence of SEQ ID NO:53 or SEQ ID NO:62, and (iii) functional variants of the peptides of (i) and (ii). In certain embodiment of the foregoing methods, the EphA3 HLA class I-binding peptide and the EphA3 HLA class II-binding peptide are combined as a polytope polypeptide. In still other embodiments, the EphA3 HLA class II binding peptide comprises an endosomal targeting signal, preferably an endosomal targeting portion of human invariant chain Ii or LAMP-1.

According to yet another aspect of the invention, methods for treating a subject having a disorder characterized by expression of EphA3 are provided. The methods include administering to the subject an amount of an agent which enriches selectively in the subject the presence of complexes of an HLA class II molecule and an EphA3 HLA class II-binding peptide, sufficient to ameliorate the disorder. Preferably the HLA class II molecule is an HLA-DR11 molecule, such as HLA-DRB1\*1101. In certain embodiments, the EphA3 HLA class II-binding peptide is a fragment of the amino acid sequence of SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, or a functional variant thereof. In certain embodiments, the agent comprises an EphA3 HLA class II binding peptide, which preferably is selected from the group consisting of (i) peptides which consist of a fragment of an amino acid sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:5 and SEQ ID NO:7, (ii) peptides which comprise the amino acid sequence of SEQ ID NO:53 or SEQ ID NO:62, and (iii) functional variants of the peptides of (i) and (ii). Preferably the EphA3 HLA class II binding peptide includes an endosomal targeting signal. Preferred endosomal targeting signals include endosomal targeting portions of human invariant chain Ii or LAMP-1.

Additional methods for treating a subject having a disorder characterized by expression of EphA3 are provided in another aspect of the invention. The methods include

administering to the subject an amount of autologous CD4<sup>+</sup> T lymphocytes sufficient to ameliorate the disorder, wherein the CD4<sup>+</sup> T lymphocytes are specific for complexes of an HLA class II molecule and an EphA3 HLA class II-binding peptide. Preferably the HLA class II molecule is an HLA-DR11 molecule, such as HLA-DRB1\*1101. In certain  
5 embodiments, the EphA3 HLA class II-binding peptide is selected from the group consisting of (i) peptides which consist of a fragment of an amino acid sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:5 and SEQ ID NO:7, (ii) peptides which comprise the amino acid sequence of SEQ ID NO:53 or SEQ ID NO:62, and (iii) functional variants of the peptides of (i) and (ii).

10 According to another aspect of the invention, an isolated polypeptide is provided. The isolated polypeptide binds selectively an EphA3 HLA class II-binding peptide, provided that the isolated polypeptide is not an HLA class II molecule. In certain embodiments, the isolated polypeptide is an antibody and preferably is a monoclonal antibody, especially a chimeric or humanized antibody. In other embodiments, the isolated polypeptide is an antibody fragment  
15 selected from the group consisting of a Fab fragment, a F(ab)<sub>2</sub> fragment or a fragment including a CDR3 region selective for an EphA3 HLA class II-binding peptide.

20 According to still another aspect of the invention, an isolated CD4<sup>+</sup> T lymphocyte is provided. The isolated CD4<sup>+</sup> T lymphocyte selectively binds a complex of an HLA class II molecule and an EphA3 HLA class II-binding peptide. Preferably the HLA class II molecule is an HLA-DR11 molecule. In some embodiments the EphA3 HLA class II-binding peptide is selected from the group consisting of (i) peptides which consist of a fragment of an amino acid sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:5 and SEQ ID NO:7, (ii) peptides which comprise the amino acid sequence of SEQ ID NO:53 or SEQ ID NO:62, and (iii) functional variants of the peptides of (i) and (ii).

25 According to still another aspect of the invention, an isolated antigen presenting cell is provided. The isolated antigen presenting cell comprises a complex of an HLA class II molecule and an EphA3 HLA class II-binding peptide. Preferably the HLA class II molecule is an HLA-DR11 molecule. In certain embodiments the EphA3 HLA class II-binding peptide is selected from the group consisting of (i) peptides which consist of a fragment of an amino  
30 acid sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:5 and SEQ ID NO:7, (ii) peptides which comprise the amino acid sequence of SEQ ID NO:53 or SEQ ID NO:62, and (iii) functional variants of the peptides of (i) and (ii).

Methods for identifying functional variants of an EphA3 HLA class II binding peptide are provided according to another aspect of the invention. According to the methods, an EphA3 HLA class II binding peptide, an HLA class II binding molecule which binds the EphA3 HLA class II binding peptide, and a T cell which is stimulated by the EphA3 HLA class II binding peptide presented by the HLA class II binding molecule are selected. A first amino acid residue of the EphA3 HLA class II binding peptide is mutated to prepare a variant peptide. The binding of the variant peptide to HLA class II binding molecule and stimulation of the T cell are then determined, wherein binding of the variant peptide to the HLA class II binding molecule and stimulation of the T cell by the variant peptide presented by the HLA class II binding molecule indicates that the variant peptide is a functional variant. In preferred embodiments, the EphA3 HLA class II binding peptide is selected from the group consisting of (i) peptides which consist of a fragment of an amino acid sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:5 and SEQ ID NO:7, and (ii) peptides which comprise the amino acid sequence of SEQ ID NO:53 or SEQ ID NO:62. In certain embodiments, the methods further include the step of comparing the stimulation of the T cell by the EphA3 HLA class II binding peptide and the stimulation of the T cell by the functional variant as a determination of the effectiveness of the stimulation of the T cell by the functional variant.

Also included in the invention are isolated functional variants of an EphA3 HLA binding peptide identified by the foregoing method. In some embodiments, the functional variants include the amino acid sequence of SEQ ID NO:62 or a fragment thereof.

For all of the foregoing compositions and methods, where not specifically set forth, Eph HLA class I binding peptides, complexes with HLA class I molecules, nucleic acid precursors, fusion proteins, CD8<sup>+</sup> T cells, etc., and uses thereof are provided which correspond to the Eph HLA class II binding compositions and methods.

The invention also provides pharmaceutical preparations, such as vaccines, containing any one or more of the compositions described above or throughout the specification. Preferred compositions included in pharmaceutical compositions include HLA class I and/or class II binding peptides, precursors thereof, complexes of such peptides and precursors with HLA molecules, antigen presenting cells presenting such peptides and precursors, and T lymphocytes which bind such peptides and precursors. The pharmaceutical preparations can include pharmaceutically acceptable diluent carriers or excipients.

In another aspect of the invention, methods for identifying genes encoding antigens presented by MHC class II molecules are provided. The methods include providing a cDNA library in an expression plasmid containing the EBV origin of replication, cotransfecting the library and nucleic acid molecules coding for class II transactivator and for the relevant HLA class II chains of the MHC class II molecule into 293-EBNA1 cells or other cells expressing EBV nuclear antigen, contacting the cotransfected cells with a T cell, and determining the recognition of the cotransfected cells by the T cell. In certain embodiments, the step of cotransfecting further comprises cotransfecting the cells with a nucleic acid molecule coding for invariant chain Ii. In other embodiments, the step of determining the recognition comprises determining proliferation by the T cell or production of a cytokine by the T cell.

The use of the foregoing compositions, peptides and nucleic acids in the preparation of a medicament, particularly a medicament for treatment of cancer, also is provided.

These and other objects of the invention will be described in further detail in connection with the detailed description of the invention.

### **Brief Description of the Drawings**

Fig. 1 shows the stimulation of clone 19 by allogenic melanoma cell lines transfected with HLA class II constructs.

Fig. 2 shows the recognition of anti-MAGE-A3 CD4 clone 37 of transfected 293-EBNA1 cells.

Fig. 3 depicts the identification of cDNA clones encoding antigen LB33-Z.

Fig. 4 is a schematic representation of cDNA clones encoding EphA3.

Fig. 5 is a schematic drawing showing the characterization of cDNA clones and minigenes encoding antigen LB33-Z.

Fig. 6 depicts the recognition of various EphA3 and variant peptides by T cells. Fig. 6A shows SEQ ID NOs:51, 54, 53, 59 and 60 (top-to-bottom). Fig. 6B shows SEQ ID NOs:54, 61, 62 and 63 (top-to-bottom).

Fig. 7 is a graph showing expression of EphA3 in normal tissues and cells.

Fig. 8 is a graph showing expression of EphA3 in tumor tissues and cells.

### **Detailed Description of the Invention**

The invention provides isolated EphA3 peptides presented by HLA class II molecules.

which peptides stimulate the proliferation and activation of CD4<sup>+</sup> T lymphocytes. Such peptides are referred to herein as "EphA3 HLA class II binding peptides", "HLA class I or class II binding peptides", "MHC class II binding peptides", "HLA binding peptides", and the like. Hence, one aspect of the invention is an isolated peptide which is a fragment of the EphA3 amino acid sequence (SEQ ID NOS: 3, 5 or 7). In another aspect, the HLA class II binding peptides include the amino acid sequence of SEQ ID NO:53.

The examples below show the identification of clones encoding peptides which are EphA3 HLA class II binding peptides. These exemplary peptides are processed translation products of the nucleic acid of SEQ ID NOs:2, 4 or 6. As such, it will be appreciated by one of ordinary skill in the art that the translation products from which an EphA3 HLA class II binding peptide is processed to a final form for presentation may be of any length or sequence so long as they encompass the EphA3 HLA class II binding peptide. Peptides or proteins as small as 9 amino acids and as large as the amino acid sequence of the EphA3 protein are appropriately processed, presented by HLA molecules and effective in stimulating T lymphocytes. EphA3 HLA binding peptides may have one, two, three, four, five, six, seven, eight, nine, ten, or more amino acids added to either or both ends. The added amino acids can be related (e.g. flanking amino acids from EphA3) or unrelated to the EphA3 peptide. The antigenic portion of such a peptide is cleaved out under physiological conditions for presentation by HLA molecules.

It is also well known in the art that HLA class II peptide length is variable between about 10 amino acids and about 30 amino acids (Engelhard, *Ann. Rev. Immunol.* 12:181-201, 1994). Most of the HLA class II binding peptides fall in to the length range of 12-19 amino acids. Nested sets of HLA class II binding peptides have been identified, wherein the peptides share a core sequence but have different amino acids at amino and/or carboxyl terminal ends (see, e.g., Chiczy et al., *J. Exp. Med.* 178:27-47, 1993). Thus additional EphA3 HLA class II binding peptides, as well as Eph family HLA class II binding peptides, can be identified by one of ordinary skill in the art according to the procedures described herein.

The EphA3 polypeptide also may contain HLA class I binding peptides, which may be tumor rejection antigens. A tumor rejection antigen is an example of a fragment of a tumor associated polypeptide which retains the functional capability of HLA binding and interaction with T lymphocytes. Tumor rejection antigens presented by HLA class I molecules typically are 9 amino acids in length, although peptides of 8, 9 and 10 and more amino acids also retain

the capability to interact with HLA and T lymphocytes to an extent effective to provoke a cytotoxic T lymphocyte response (see, e.g., Van den Eynde & Brichard, *Curr. Opin. Immunol.* 7:674-681, 1995; Coulie et al., *Stem Cells* 13:393-403, 1995).

Thus, an EphA3 HLA binding peptide which is a fragment of EphA3 is any portion of EphA3 which contains an HLA binding peptide. For example, if the HLA binding peptide has a minimal length of 8 amino acids, the invention embraces any portion of EphA3 which contains the 8 amino acid sequence. Thus, in this example, the EphA3 HLA binding peptide can be a fragment of EphA3 having 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 amino acids, and so on, up to the entire length of EphA3 less one amino acid, embracing every integer therebetween.

The procedures described in the Examples can be utilized to identify Eph family HLA class I and/or II binding peptides, particularly those derived from EphA3. Thus, for example, one can load antigen presenting cells, such as dendritic cells of normal blood donors, with a recombinant Eph protein (or a fragment thereof) by contacting the cells with the Eph polypeptide or by introducing into the cells a nucleic acid molecule which directs the expression of the Eph protein of interest. The antigen-presenting cells then can be used to induce *in vitro* the activation and proliferation of specific CD4 lymphocytes which recognize Eph HLA class II binding peptides. The sequence of the peptides then can be determined as described in the Examples, e.g., by stimulating cells with peptide fragments of the Eph protein used to stimulate the activation and proliferation of CD4 lymphocytes. Alternatively, one can load antigen presenting cells with peptides derived from a Eph protein such as EphA3. For example, one can make predictions of peptide sequences derived from Eph family proteins which are candidate HLA class I or class II binding peptides based on the consensus amino acid sequences for binding HLA class I or class II molecules. In this regard, see, e.g. International applications PCT/US96/03182 and PCT/US98/01373. Peptides which are thus selected can be used in the assays described herein for inducing specific T lymphocytes and identification of peptides. Additional methods of selecting and testing peptides for HLA class I and class II binding are well known in the art and are described elsewhere herein. Several HLA class II binding peptides are presented in the Examples.

As noted above, the invention embraces functional variants of EphA3 HLA class II binding peptides. As used herein, a "functional variant" or "variant" of a HLA class I or class II binding peptide is a peptide which contains one or more modifications to the primary amino

acid sequence of a HLA class I or class II binding peptide and retains the HLA class I or class II and T cell receptor binding properties disclosed herein. Preferred HLA binding peptide functional variants have 6 or fewer amino acid modifications (e.g., deletions, additions or substitutions) relative to the EphA3 amino acid sequence. More preferred functional variants have 4 or fewer amino acid modifications, and most preferred functional variants have 2 or fewer amino acid modifications relative to the EphA3 amino acid sequence. Preferably the modifications are made to amino acids which do not serve as anchor residues for HLA binding. SEQ ID NO:62 represents one example of a functional variant which retains the HLA class II binding properties of the normal EphA3 peptide.

Modifications which create an EphA3 HLA binding peptide functional variant can be made for example 1) to enhance a property of an EphA3 HLA binding peptide, such as peptide stability in an expression system or the stability of protein-protein binding such as HLA-peptide binding; 2) to provide a novel activity or property to an EphA3 HLA binding peptide, such as addition of an antigenic epitope or addition of a detectable moiety; or 3) to provide a different amino acid sequence that produces the same or similar T cell stimulatory properties. Modifications to EphA3 (as well as Eph family) HLA class I or class II binding peptides can be made to nucleic acids which encodes the peptide, and can include deletions, point mutations, truncations, amino acid substitutions and additions of amino acids. Alternatively, modifications can be made directly to the polypeptide, such as by cleavage, addition of a linker molecule, addition of a detectable moiety, such as biotin, addition of a fatty acid, substitution of one amino acid for another and the like.

One of skill in the art will be familiar with methods for predicting the effect on protein conformation of a change in protein sequence, and can thus "design" a variant EphA3 HLA binding peptide according to known methods. One example of such a method is described by Dahiyat and Mayo (*Science* 278:82-87, 1997), whereby proteins can be designed *de novo*. The method can be applied to a known polypeptide to vary a only a portion of the amino acid sequence. By applying the computational methods of Dahiyat and Mayo, specific variants of an EphA3 HLA binding peptide can be proposed and tested to determine whether the variant retains a desired conformation, i.e., is capable of binding HLA.

Variants also can be selected from libraries of peptides, which can be random peptides or peptides based on the sequence of the Eph peptides including substitutions at one or more positions. For example, a peptide library can be used in competition assays with complexes

of Eph peptides bound to HLA class II molecules (e.g. dendritic cells loaded with Eph peptide). Peptides which compete for binding of the Eph peptide to HLA class I or class II molecule can be sequenced and used in other assays (e.g. T lymphocyte proliferation) to determine suitability as Eph peptide functional variants.

5        Modifications also embrace fusion proteins comprising all or part of a Eph HLA class I or class II binding peptide amino acid sequence, such as the invariant chain-EphA3 fusion proteins described herein. The invention thus embraces fusion proteins comprising EphA3 HLA class II binding peptides and optionally endosomal targeting signals such as the human invariant chain (Ii) or LAMP-1. Fusion of an endosomal targeting portion of the human  
10        invariant chain to a protein can result in efficient targeting of the protein to the HLA class II peptide presentation pathway. An "endosomal targeting portion" of the human invariant chain or other targeting polypeptide is that portion of the molecule which, when fused or conjugated to a second polypeptide, increases endosomal localization of the second polypeptide. Thus endosomal targeting portions can include the entire sequence or only a  
15        small portion of a targeting polypeptide such as human invariant chain Ii. One of ordinary skill in the art can readily determine an endosomal targeting portion of a targeting molecule. Additional endosomal targeting signals can be identified by one of ordinary skill in the art, fused to EphA3 or an EphA3 HLA class II binding portion thereof, and tested for targeting to the HLA class II peptide presentation pathway using no more than routine experimentation.

20        The amino acid sequence of Eph HLA binding peptides may be of natural or non-natural origin, that is, they may comprise a natural Eph HLA binding peptide molecule or may comprise a modified sequence as long as the amino acid sequence retains the ability to stimulate T cells when presented and retains the property of binding to an HLA molecule. For example, EphA3 HLA class II binding peptides in this context may be fusion proteins  
25        including an EphA3 HLA class II binding peptide and unrelated amino acid sequences, synthetic peptides of EphA3 fragment amino acid sequences, labeled peptides, peptides isolated from patients with an EphA3 expressing cancer, peptides isolated from cultured cells which express EphA3, peptides coupled to nonpeptide molecules (for example in certain drug delivery systems) and the like.

30        Preferably, Eph HLA binding peptides are non-hydrolyzable. To provide such peptides, one may select Eph HLA binding peptides from a library of non-hydrolyzable peptides, such as peptides containing one or more D-amino acids or peptides containing one or

more non-hydrolyzable peptide bonds linking amino acids. Alternatively, one can select peptides which are optimal for inducing CD4<sup>+</sup> T lymphocytes (for class II binding peptides) or CD8<sup>+</sup> T lymphocytes (for class I binding peptides) and then modify such peptides as necessary to reduce the potential for hydrolysis by proteases. For example, to determine the susceptibility to proteolytic cleavage, peptides may be labeled and incubated with cell extracts or purified proteases and then isolated to determine which peptide bonds are susceptible to proteolysis, e.g., by sequencing peptides and proteolytic fragments. Alternatively, potentially susceptible peptide bonds can be identified by comparing the amino acid sequence of an EphA3 HLA binding peptide with the known cleavage site specificity of a panel of proteases. Based on the results of such assays, individual peptide bonds which are susceptible to proteolysis can be replaced with non-hydrolyzable peptide bonds by *in vitro* synthesis of the peptide.

Many non-hydrolyzable peptide bonds are known in the art, along with procedures for synthesis of peptides containing such bonds. Non-hydrolyzable bonds include -psi[CH<sub>2</sub>NH]-reduced amide peptide bonds, -psi[COCH<sub>2</sub>]-ketomethylene peptide bonds, -psi[CH(CN)NH]-(cyanomethylene)amino peptide bonds, -psi[CH<sub>2</sub>CH(OH)]-hydroxyethylene peptide bonds, -psi[CH<sub>2</sub>O]-peptide bonds, and -psi[CH<sub>2</sub>S]-thiomethylene peptide bonds.

Nonpeptide analogs of peptides, e.g., those which provide a stabilized structure or lessened biodegradation, are also contemplated. Peptide mimetic analogs can be prepared based on a selected EphA3 HLA binding peptide by replacement of one or more residues by nonpeptide moieties. Preferably, the nonpeptide moieties permit the peptide to retain its natural conformation, or stabilize a preferred, e.g., bioactive, conformation. Such peptides can be tested in molecular or cell-based binding assays to assess the effect of the substitution(s) on conformation and/or activity. One example of methods for preparation of nonpeptide mimetic analogs from peptides is described in Nachman et al., *Regul. Pept.* 57:359-370 (1995). Peptide as used herein embraces all of the foregoing.

Variants of EphA3 HLA binding peptides can be prepared by substituting one or more amino acids of the binding peptide, preferably in accordance with known conserved residues for HLA binding. If a variant involves a change to an amino acid of an EphA3 polypeptide fragment, functional variants of the EphA3 HLA binding peptide having conservative amino acid substitutions typically will be preferred, i.e., substitutions which retain a property of the original amino acid such as charge, hydrophobicity, conformation, etc. Examples of

conservative substitutions of amino acids include substitutions made amongst amino acids within the following groups: (a) M, I, L, V; (b) F, Y, W; (c) K, R, H; (d) A, G; (e) S, T; (f) Q, N; and (g) E, D. However, nonconservative substitutions can also be made to the amino acid sequence to prepare functional variants. For example, the functional variant peptide presented below (SEQ ID NO:62) has a nonconservative Cys-to-Ser mutation.

Peptides presented by certain MHC/HLA molecules generally conform to motifs. These motifs are known in some cases, and can be used to screen Eph proteins for the presence of potential class I and/or class II peptides. Summaries of class I and class II motifs have been published (e.g., Rammensee et al., *Immunogenetics* 41:178-228, 1995). As for HLA-class I-restricted peptides, HLA-class II-restricted peptides have preferred anchor residues within a HLA binding core of 9 to 10 amino acids. (See, e.g., Rammensee et al., MHC Ligands and Peptide Motifs, Molecular Biology Intelligence Unit, Springer, 1997).

One also can search for class I and class II motifs using computer algorithms. For example, computer programs for predicting potential CTL epitopes based on known class I motifs have been described (see, e.g., Parker et al., *J. Immunol.* 152:163, 1994; D'Amato et al., *Human Immunol.* 43:13-18, 1995; Drijfhout et al., *Human Immunol.* 43:1-12, 1995). HLA binding predictions can conveniently be made using an algorithm available via the Internet on the National Institutes of Health World Wide Web site at URL <http://bimas.dcrt.nih.gov>. See also the website of: SYFPEITHI: An Internet Database for MHC Ligands and Peptide Motifs (access via <http://www.uni-tuebingen.de/uni/kxi/> or <http://134.2.96.221/scripts/hlaserver.dll/EpPredict.htm>).

Other methods for identifying functional variants of the EphA3 HLA class II binding peptides are provided in a published PCT application of Strominger and Wucherpennig (PCT/US96/03182). These methods rely upon the development of amino acid sequence motifs to which potential epitopes may be compared. Each motif describes a finite set of amino acid sequences in which the residues at each (relative) position may be (a) restricted to a single residue, (b) allowed to vary amongst a restricted set of residues, or (c) allowed to vary amongst all possible residues. For example, a motif might specify that the residue at a first position may be any one of the residues valine, leucine, isoleucine, methionine, or phenylalanine; that the residue at the second position must be histidine; that the residue at the third position may be any amino acid residue; that the residue at the fourth position may be any one of the residues valine, leucine, isoleucine, methionine, phenylalanine, tyrosine or

tryptophan; and that the residue at the fifth position must be lysine.

Sequence motifs for EphA3 HLA class II binding peptide functional variants can be developed by analysis of the binding domains or binding pockets of major histocompatibility complex HLA-DR proteins and/or the T cell receptor ("TCR") contact points of the EphA3 HLA class II binding peptides disclosed herein. By providing a detailed structural analysis of the residues involved in forming the HLA class II binding pockets, one is enabled to make predictions of sequence motifs for binding of Eph peptides to any of the HLA class II proteins.

Using these sequence motifs as search, evaluation, or design criteria, one is enabled to identify classes of peptides (e.g. Eph HLA class II binding peptides, particularly the EphA3 peptides disclosed herein, and functional variants thereof) which have a reasonable likelihood of binding to a particular HLA molecule and of interacting with a T cell receptor to induce T cell response. These peptides can be synthesized and tested for activity as described herein. Use of these motifs, as opposed to pure sequence homology (which excludes many peptides which are antigenically similar but quite distinct in sequence) or sequence homology with unlimited "conservative" substitutions (which admits many peptides which differ at critical highly conserved sites), represents a method by which one of ordinary skill in the art can evaluate peptides for potential application in the treatment of disease.

The Strominger and Wucherpennig PCT application, and references cited therein, all of which are incorporated by reference, describe the HLA class II and TCR binding pockets which contact residues of an HLA class II peptide. By keeping the residues which are likely to bind in the HLA class II and/or TCR binding pockets constant or permitting only specified substitutions, functional variants of Eph HLA class II binding peptides can be prepared which retain binding to HLA class II and T cell receptor.

Thus methods for identifying additional Eph family HLA class I and/or class II peptides, in particular EphA3 HLA class II binding peptides, and functional variants thereof, are provided. In general, any Eph protein can be subjected to the analysis noted above, peptide sequences selected and the tested as described herein. Eph family proteins include, for example, those proteins having substantial amino acid identity with EphA3. With respect to EphA3, for example, the methods include selecting an EphA3 HLA class II binding peptide, an HLA class II binding molecule which binds the EphA3 HLA class II binding peptide, and a T cell which is stimulated by the EphA3 HLA class II binding peptide

presented by the HLA class II binding molecule. In preferred embodiments, the EphA3 HLA class II binding peptide comprises a fragment the amino acid sequence of SEQ ID NO:3, SEQ ID NO:5, or SEQ ID NO:7, particularly fragment which include the amino acids of SEQ ID NO:53. A first amino acid residue of the EphA3 HLA class II binding peptide is mutated to  
5 prepare a variant peptide. The amino acid residue can be mutated according to the principles of HLA and T cell receptor contact points set forth in the Strominger and Wuchterpfennig PCT application described above. Any method for preparing variant peptides can be employed, such as synthesis of the variant peptide, recombinantly producing the variant peptide using a mutated nucleic acid molecule, and the like.

10 The binding of the variant peptide to HLA class II binding molecule and stimulation of the T cell are then determined according to standard procedures. For example, as exemplified below, the variant peptide can be contacted with an antigen presenting cell which contains the HLA class II molecule which binds the EphA3 peptide to form a complex of the variant peptide and antigen presenting cell. Similarly, the antigen presenting cell can be  
5 transfected with a nucleic acid molecule that encodes and expresses the variant peptide. This complex can then be contacted with a T cell which recognizes the EphA3 HLA class II binding peptide presented by the HLA class II binding molecule (e.g., T cell clone 19). T cells can be obtained from a patient having a condition characterized by expression of EphA3. Recognition of variant peptides by the T cells can be determined by measuring an indicator of  
20 T cell stimulation such as TNF or IFN $\gamma$  production. Similar procedures can be carried out for identification and characterization of other Eph family HLA class II binding peptides, as well as HLA class I binding peptides.

Binding of a variant peptide to the HLA class I or II binding molecule and stimulation of the T cell by the variant peptide presented by the HLA class I or II binding molecule  
25 indicates that the variant peptide is a functional variant. The methods also can include the step of comparing the stimulation of the T cell by the EphA3 HLA binding peptide and the stimulation of the T cell by the functional variant as a determination of the effectiveness of the stimulation of the T cell by the functional variant. By comparing the functional variant with the EphA3 HLA binding peptide, peptides with increased T cell stimulatory properties can be  
30 prepared.

Variants of the EphA3 HLA binding peptides prepared by any of the foregoing methods can be sequenced, if necessary, to determine the amino acid sequence and thus

deduce the nucleotide sequence which encodes such variants.

Also a part of the invention are those nucleic acid sequences which code for a Eph HLA binding peptides or variant thereof and other nucleic acid sequences which hybridize to a nucleic acid molecule consisting of the above described nucleotide sequences, under stringent conditions. The term "stringent conditions" as used herein refers to parameters with which the art is familiar. Nucleic acid hybridization parameters may be found in references which compile such methods, e.g. *Molecular Cloning: A Laboratory Manual*, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, or *Current Protocols in Molecular Biology*, F.M. Ausubel, et al., eds., John Wiley & Sons, Inc., New York. More specifically, stringent conditions, as used herein, refers to hybridization at 65°C in hybridization buffer (3.5 x SSC, 0.02% Ficoll, 0.02% Polyvinyl pyrrolidone, 0.02% Bovine Serum Albumin, 25mM NaH<sub>2</sub>PO<sub>4</sub> (pH7), 0.5% SDS, 2mM EDTA). SSC is 0.15M Sodium Chloride/0.15M Sodium Citrate, pH 7; SDS is Sodium Dodecyl Sulphate; and EDTA is Ethylene diaminetetraacetic acid. After hybridization, the membrane upon which the DNA is transferred is washed at 2xSSC at room temperature and then at 0.1 - 0.5xSSC/0.1xSDS at temperatures up to 68°C.

There are other conditions, reagents, and so forth which can be used, which result in a similar degree of stringency. The skilled artisan will be familiar with such conditions, and thus they are not given here. It will be understood, however, that the skilled artisan will be able to manipulate the conditions in a manner to permit the clear identification of homologs and alleles of nucleic acids encoding the Eph HLA class II binding peptides of the invention. The skilled artisan also is familiar with the methodology for screening cells and libraries for expression of such molecules which then are routinely isolated, followed by isolation of the pertinent nucleic acid molecule and sequencing.

In general homologs and alleles typically will share at least 90% amino acid identity and/or at least 75% nucleotide identity to the amino acid sequence of an EphA3 HLA class II binding peptide (such as fragments of SEQ ID NO:3, SEQ ID NO:5, and SEQ ID NO:7) or nucleic acids which encode such a peptide, respectively. In some instances homologs and alleles will share at least 90% nucleotide identity and/or at least 95% amino acid identity and in still other instances will share at least 95% nucleotide identity and/or at least 99% amino acid identity. Complements of the foregoing nucleic acids also are embraced by the invention.

In screening for nucleic acids which encode a Eph HLA class II binding peptide, a nucleic acid hybridization such as a Southern blot or a Northern blot may be performed using the foregoing conditions, together with a  $^{32}\text{P}$  probe. After washing the membrane to which DNA encoding a Eph HLA class II binding peptide is finally transferred, the membrane can  
5 be placed against X-ray film to detect the radioactive signal.

The invention also includes the use of nucleic acid sequences which include alternative codons that encode the same amino acid residues of the Eph HLA binding peptides. For example, leucine residues can be encoded by the codons CUA, CUC, CUG, CUU, UUA and UUG. Each of the six codons is equivalent for the purposes of encoding a  
10 leucine residue. Thus, it will be apparent to one of ordinary skill in the art that any of the leucine-encoding nucleotide triplets may be employed to direct the protein synthesis apparatus, *in vitro* or *in vivo*, to incorporate a leucine residue. Similarly, nucleotide sequence triplets which encode other amino acid residues comprising the EphA3 HLA binding peptides may be degenerate, such as: CGA, CGC, CGG, CGT, AGA and AGG (arginine codons);  
15 AAA and AAG (lysine codons); GUA, GUC, GUG and GUU (valine codons); GAA and GAG (glutamine codons); CAC and CAU (histidine codons); UUC and UUU (phenylalanine codons) and UAC and UAU (tyrosine codons). Other amino acid residues may be encoded similarly by multiple nucleotide sequences. Thus, the invention embraces degenerate nucleic acids that differ from the native Eph HLA binding peptides encoding nucleic acids in codon  
20 sequence due to the degeneracy of the genetic code.

The invention also provides modified nucleic acid molecules which include additions, substitutions and deletions of one or more nucleotides. In preferred embodiments, these modified nucleic acid molecules and/or the polypeptides they encode retain at least one activity or function of the unmodified nucleic acid molecule and/or the polypeptides, such as  
25 antigenicity, enzymatic activity, receptor binding, formation of complexes by binding of peptides by MHC class I and class II molecules, etc. In certain embodiments, the modified nucleic acid molecules encode modified polypeptides, preferably polypeptides having conservative amino acid substitutions as are described elsewhere herein. The modified nucleic acid molecules are structurally related to the unmodified nucleic acid molecules and in  
30 preferred embodiments are sufficiently structurally related to the unmodified nucleic acid molecules so that the modified and unmodified nucleic acid molecules hybridize under stringent conditions known to one of skill in the art.

For example, modified nucleic acid molecules which encode polypeptides having single amino acid changes can be prepared (e.g., those encoding SEQ ID NO: 61 or 62). Each of these nucleic acid molecules can have one, two or three nucleotide substitutions exclusive of nucleotide changes corresponding to the degeneracy of the genetic code as described herein. Likewise, modified nucleic acid molecules which encode polypeptides having two amino acid changes can be prepared which have, e.g., 2-6 nucleotide changes. Numerous modified nucleic acid molecules like these will be readily envisioned by one of skill in the art, including for example, substitutions of nucleotides in codons encoding amino acids 2 and 3, 2 and 4, 2 and 5, 2 and 6, and so on. In the foregoing example, each combination of two amino acids is included in the set of modified nucleic acid molecules, as well as all nucleotide substitutions which code for the amino acid substitutions. An example of this is provided by SEQ ID NO:63. Additional nucleic acid molecules that encode polypeptides having additional substitutions (i.e., 3 or more), additions or deletions (e.g., by introduction of a stop codon or a splice site(s)) also can be prepared and are embraced by the invention as readily envisioned by one of ordinary skill in the art. Any of the foregoing nucleic acids or polypeptides can be tested by routine experimentation for retention of structural relation or activity to the nucleic acids and/or polypeptides disclosed herein.

It will also be understood that the invention embraces the use of the sequences in expression vectors, as well as to transfect host cells and cell lines, be these prokaryotic (e.g., *E. coli*), or eukaryotic (e.g., dendritic cells, CHO cells, COS cells, yeast expression systems and recombinant baculovirus expression in insect cells). The expression vectors require that the pertinent sequence, i.e., those described *supra*, be operably linked to a promoter. In addition, as it has been found that human HLA-DRB1\*1101 molecules present an EphA3 HLA class II binding peptide, the expression vector may also include a nucleic acid sequence coding for an HLA-DRB11 molecule. (For other Eph HLA class I or class II binding peptides, different HLA molecules can be used.) In a situation where the vector contains both coding sequences, it can be used to transfect a cell which does not normally express either one. The EphA3 HLA class II binding peptide coding sequence may be used alone, when, e.g. the host cell already expresses an HLA-DR11 molecule. Of course, there is no limit on the particular host cell which can be used as the vectors which contain the two coding sequences may be used in host cells which do not express HLA-DR11 molecules if desired, and the nucleic acid coding for the EphA3 HLA class II binding peptide can be used in

antigen presenting cells which express an HLA-DR11 molecule. As used herein, "an HLA-DR11 molecule" includes the subtypes HLA-DRB1\*11011, 11012, 11013, 1102, 1103, 11041, 11042, 1105, 1106, 1107, 11081, 11082, 1109, 1110, 1111, 1112, 1113, 1114, 1115, 1116, 1117, 1118, 1119, 1120, 1121, 1122, 1123, 1124, 1125, 1126, 1127, 1128, 1129, 1130, 1131, 1132, 1133, and 1134. An HLA-DR11 molecule also includes the subtypes which can be found in Bodmer et al., *Tissue Antigens* 49:297, 1996. A listing of presently identified HLA-DR11 subtypes can be found on the IMGT/HLA database at internet URL <http://www.ebi.ac.uk/imgt/hla/>.

As used herein, a "vector" may be any of a number of nucleic acids into which a desired sequence may be inserted by restriction and ligation for transport between different genetic environments or for expression in a host cell. Vectors are typically composed of DNA although RNA vectors are also available. Vectors include, but are not limited to, plasmids, phagemids and virus genomes. A cloning vector is one which is able to replicate autonomously or after integration into the genome in a host cell, and which is further characterized by one or more endonuclease restriction sites at which the vector may be cut in a determinable fashion and into which a desired DNA sequence may be ligated such that the new recombinant vector retains its ability to replicate in the host cell. In the case of plasmids, replication of the desired sequence may occur many times as the plasmid increases in copy number within the host bacterium or just a single time per host before the host reproduces by mitosis. In the case of phage, replication may occur actively during a lytic phase or passively during a lysogenic phase. An expression vector is one into which a desired DNA sequence may be inserted by restriction and ligation such that it is operably joined to regulatory sequences and may be expressed as an RNA transcript. Vectors may further contain one or more marker sequences suitable for use in the identification of cells which have or have not been transformed or transfected with the vector. Markers include, for example, genes encoding proteins which increase or decrease either resistance or sensitivity to antibiotics or other compounds, genes which encode enzymes whose activities are detectable by standard assays known in the art (e.g.,  $\beta$ -galactosidase, alkaline phosphatase or luciferase), and genes which visibly affect the phenotype of transformed or transfected cells, hosts, colonies or plaques (e.g., green fluorescent protein). Preferred vectors are those capable of autonomous replication and expression of the structural gene products present in the DNA segments to which they are operably joined.

For stimulation of CD4<sup>+</sup> T cells, the expression vectors can contain sequences which target an Eph family polypeptide, e.g. EphA3, or a HLA class II binding peptide derived therefrom, to the endosomes of a cell in which the protein or peptide is expressed. As described below in the Examples, this may not be necessary in all cases, and in fact it may be preferred to forego fusing a endosomal targeting sequence to the peptide. It is also possible to express invariant chain separately to increase presentation by HLA class II molecules. HLA class II molecules contain an invariant chain (Ii) which impedes binding of other molecules to the HLA class II molecules. This invariant chain is cleaved in endosomes, thereby permitting binding of peptides by HLA class II molecules. Therefore it may be preferable that the EphA3 HLA class II binding peptides and precursors thereof (e.g. the EphA3 protein) are targeted to the endosome, thereby enhancing EphA3 HLA class II binding peptide binding to HLA class II molecules. Targeting signals for directing molecules to endosomes are known in the art and these signals conveniently can be incorporated in expression vectors such that fusion proteins which contain the endosomal targeting signal are produced. Sanderson et al. (*Proc. Nat'l. Acad. Sci. USA* 92:7217-7221, 1995), Wu et al. (*Proc. Nat'l. Acad. Sci. USA* 92:11671-11675, 1995) and Thomson et al. (*J. Virol.* 72:2246-2252, 1998) describe endosomal targeting signals (including invariant chain Ii and lysosomal-associated membrane protein LAMP-1) and their use in directing antigens to endosomal and/or lysosomal cellular compartments.

Endosomal targeting signals such as invariant chain also can be conjugated to EphA3 protein or peptides by non-peptide bonds (i.e. not fusion proteins) to prepare a conjugate capable of specifically targeting EphA3. Specific examples of covalent bonds include those wherein bifunctional cross-linker molecules are used. The cross-linker molecules may be homobifunctional or heterobifunctional, depending upon the nature of the molecules to be conjugated. Homobifunctional cross-linkers have two identical reactive groups. Heterobifunctional cross-linkers are defined as having two different reactive groups that allow for sequential conjugation reaction. Various types of commercially available cross-linkers are reactive with one or more of the following groups; primary amines, secondary amines, sulfhydryls, carboxyls, carbonyls and carbohydrates. One of ordinary skill in the art will be able to ascertain without undue experimentation the preferred molecule for linking the endosomal targeting moiety and EphA3 peptide or protein, based on the chemical properties of the molecules being linked and the preferred characteristics of the bond or bonds.

As used herein, a coding sequence and regulatory sequences are said to be "operably" joined when they are covalently linked in such a way as to place the expression or transcription of the coding sequence under the influence or control of the regulatory sequences. If it is desired that the coding sequences be translated into a functional protein, two DNA sequences are said to be operably joined if induction of a promoter in the 5' regulatory sequences results in the transcription of the coding sequence and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region to direct the transcription of the coding sequences, or (3) interfere with the ability of the corresponding RNA transcript to be translated into a protein. Thus, a promoter region would be operably joined to a coding sequence if the promoter region were capable of effecting transcription of that DNA sequence such that the resulting transcript might be translated into the desired protein or polypeptide.

The precise nature of the regulatory sequences needed for gene expression may vary between species or cell types, but shall in general include, as necessary, 5' non-transcribed and 5' non-translated sequences involved with the initiation of transcription and translation respectively, such as a TATA box, capping sequence, CAAT sequence, and the like. Especially, such 5' non-transcribed regulatory sequences will include a promoter region which includes a promoter sequence for transcriptional control of the operably joined gene. Regulatory sequences may also include enhancer sequences or upstream activator sequences as desired. The vectors of the invention may optionally include 5' leader or signal sequences. The choice and design of an appropriate vector is within the ability and discretion of one of ordinary skill in the art.

Expression vectors containing all the necessary elements for expression are commercially available and known to those skilled in the art. See, e.g., Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, 1989. Cells are genetically engineered by the introduction into the cells of heterologous DNA (RNA) encoding an EphA3 HLA class II binding peptide. That heterologous DNA (RNA) is placed under operable control of transcriptional elements to permit the expression of the heterologous DNA in the host cell. As described herein, such expression constructs optionally also contain nucleotide sequences which encode endosomal targeting signals, preferably human invariant chain or a targeting fragment thereof, or LAMP-1.

Preferred systems for mRNA expression in mammalian cells are those such as pRc/CMV (available from Invitrogen, Carlsbad, CA) that contain a selectable marker such as a gene that confers G418 resistance (which facilitates the selection of stably transfected cell lines) and the human cytomegalovirus (CMV) enhancer-promoter sequences. Additionally, suitable for expression in primate or canine cell lines is the pCEP4 vector (Invitrogen), which contains an Epstein Barr virus (EBV) origin of replication, facilitating the maintenance of plasmid as a multicopy extrachromosomal element. Another expression vector is the pEF-BOS plasmid containing the promoter of polypeptide Elongation Factor 1 $\alpha$ , which stimulates efficiently transcription *in vitro*. The plasmid is described by Mishizuma and Nagata (*Nuc. Acids Res.* 18:5322, 1990), and its use in transfection experiments is disclosed by, for example, Demoulin (*Mol. Cell. Biol.* 16:4710-4716, 1996). Still another preferred expression vector is an adenovirus, described by Stratford-Perricaudet, which is defective for E1 and E3 proteins (*J. Clin. Invest.* 90:626-630, 1992). The use of the adenovirus as an Adeno.P1A recombinant is disclosed by Warnier et al., in intradermal injection in mice for immunization against P1A (*Int. J. Cancer*, 67:303-310, 1996).

The invention also embraces so-called expression kits, which allow the artisan to prepare a desired expression vector or vectors. Such expression kits include at least separate portions of at least two of the previously discussed materials. Other components may be added, as desired.

The invention as described herein has a number of uses, some of which are described herein. The following uses are described for EphA3 HLA class I or II binding peptides but are equally applicable to use of other Eph family HLA class I or II binding peptides. First, the invention permits the artisan to diagnose a disorder characterized by expression of an EphA3 HLA class I or II binding peptide. These methods involve determining expression of an EphA3 HLA class I or II binding peptide, or a complex of an EphA3 HLA class II binding peptide and an HLA class II molecule in a biological sample. The expression of a peptide or complex of peptide and HLA class I or II molecule can be determined by assaying with a binding partner for the peptide or complex, such as an antibody.

The invention also permits the artisan to treat a subject having a disorder characterized by expression of an EphA3 HLA class I or II binding peptide. Treatments include administering an agent which enriches in the subject a complex of an EphA3 HLA class I or II binding peptide and an HLA class I and II molecule, and administering T lymphocytes which

are specific for such complexes. Agents useful in the foregoing treatments include EphA3 HLA class I or II binding peptides and functional variants thereof, endosome-targeted fusion proteins which include such EphA3 peptides, nucleic acids which express such proteins and peptides (including viruses which contain the nucleic acids), complexes of such peptides and HLA class I or II binding molecules (e.g. HLA DRB1\*1101), antigen presenting cells bearing complexes of an EphA3 HLA class I or II binding peptide and an HLA class I or II binding molecule, and the like.

The invention also permits one to selectively enrich a population of T lymphocytes for CD8<sup>+</sup> or CD4<sup>+</sup> T lymphocytes specific for an EphA3 HLA class I or II binding peptide. Such methods include contacting a source of T lymphocytes with an agent presenting a complex of the EphA3 HLA class II-binding peptide and an HLA class II molecule in an amount sufficient to selectively enrich the population of T lymphocytes with the CD4<sup>+</sup> T lymphocytes. Preferably the source of T lymphocytes is a tissue, such as lymph nodes, or a population of cells which contains T lymphocytes, such as peripheral blood lymphocytes. Other sources of T lymphocytes are known to one of ordinary skill in the art. In certain embodiments the source of T lymphocytes is an isolated population of T lymphocytes. Preferably the sources and/or populations of T lymphocytes are isolated. An agent as used in this context includes the various antigen presenting molecules and cells known to one of ordinary skill in the art, including without limitation dendritic cells, cells transfected with HLA molecules, tetramers of HLA molecules and antigen, etc.

As used herein with respect to cells, polypeptide molecules and/or nucleic acid molecules, "isolated" means separated from its native environment and present in sufficient quantity to permit its identification or use. When referring to cells, isolated means, for example: (i) separated, as by histological type or physical processes such as centrifugation, (ii) amplified, as by *in vitro* expansion, or (iii) purified, as by immunological recognition of cell surface molecules. When referring to a "protein," "peptide" or "polypeptide", isolated means, for example : (i) selectively produced by expression of a recombinant nucleic acid or (ii) purified as by chromatography or electrophoresis. When referring to a nucleic acid molecule, the term "isolated" means, for example: (i) amplified *in vitro* by, for example, polymerase chain reaction (PCR); (ii) recombinantly produced by cloning; (iii) purified, as by cleavage and gel separation; or (iv) synthesized by, for example, chemical synthesis. An isolated nucleic acid is one which is readily manipulable by recombinant DNA techniques well known

in the art. Thus, a nucleotide sequence contained in a vector in which 5' and 3' restriction sites are known or for which polymerase chain reaction (PCR) primer sequences have been disclosed is considered isolated but a nucleic acid sequence existing in its native state in its natural host is not.

5 Isolated cells, polypeptide molecules and nucleic acid molecules may, but need not be, substantially pure. The term "substantially pure" means that the cells, polypeptide molecules and nucleic acid molecules are essentially free of other substances with which they may be found in nature or *in vivo* systems to an extent practical and appropriate for their intended use. Substantially pure cells, polypeptide molecules and nucleic acid molecules may be produced  
10 by techniques well known in the art. Because isolated cells, polypeptide molecules and/or nucleic acid molecules may be admixed with a pharmaceutically acceptable carrier in a pharmaceutical preparation, the cells, polypeptide molecules and/or nucleic acid molecules may comprise only a small percentage by weight of the preparation. The cells, polypeptide molecules and/or nucleic acid molecules are nonetheless isolated in that they have been  
15 separated from the substances with which they may be associated in living systems, i.e. isolated from other cells, polypeptide molecules and/or nucleic acid molecules.

The identification of the EphA3 HLA class I or II binding peptides also makes it possible to isolate nucleic acids which encode these binding peptides. Nucleic acids can be used to produce *in vitro* or in prokaryotic or eukaryotic host cells the EphA3 HLA class I or II  
20 binding peptides. A variety of methodologies well-known to the skilled practitioner can be utilized to obtain isolated EphA3 HLA class I or II binding peptides. For example, an expression vector may be introduced into cells to cause production of the peptides. In another method, mRNA transcripts may be microinjected or otherwise introduced into cells to cause production of the encoded peptides. Translation of mRNA in cell-free extracts such as the  
25 reticulocyte lysate system also may be used to produce peptides. Peptides comprising the EphA3 HLA class I or II binding peptide of the invention may also be synthesized *in vitro*. Those skilled in the art also can readily follow known methods for isolating peptides in order to obtain isolated EphA3 HLA class I or II binding peptides. These include, but are not limited to, immunochromatography, HPLC, size-exclusion chromatography, ion-exchange  
30 chromatography and immune-affinity chromatography.

These isolated EphA3 HLA class I or II binding peptides, proteins which include such peptides, or complexes of the peptides and HLA class I or II molecules, such as an HLA-

DRB1\*1101 molecule, may be combined with materials such as adjuvants to produce vaccines useful in treating disorders characterized by expression of EphA3 HLA binding peptides. In addition, vaccines can be prepared from cells which present the EphA3 HLA binding peptide/HLA complexes on their surface, such as dendritic cells, B cells, non-proliferative transfectants, etcetera. In all cases where cells are used as a vaccine, these can be cells transfected with coding sequences for one or both of the components necessary to stimulate T lymphocytes, or be cells which already express both molecules without the need for transfection. For example, autologous antigen presenting cells can be isolated from a patient and treated to obtain cells which present EphA3 epitopes in association with HLA class I and HLA class II molecules. These cells would be capable of stimulating both CD4<sup>+</sup> and CD8<sup>+</sup> cell responses. Such antigen presenting cells can be obtained, for example, by infecting dendritic cells with recombinant viruses encoding an Ii.EphA3 fusion protein. Dendritic cells also can be loaded with HLA class I and HLA class II epitopes.

Vaccines also encompass naked DNA or RNA, encoding an EphA3 HLA binding peptide or precursor thereof, which may be produced *in vitro* and administered via injection, particle bombardment, nasal aspiration and other methods. Vaccines of the "naked nucleic acid" type have been demonstrated to provoke an immunological response including generation of CTLs specific for the peptide encoded by the naked nucleic acid (*Science* 259:1745-1748, 1993). Vaccines also include nucleic acids packaged in a virus, liposome or other particle, including polymeric particles useful in drug delivery.

The immune response generated or enhanced by any of the treatments described herein can be monitored by various methods known in the art. For example, the presence of T cells specific for a given antigen can be detected by direct labeling of T cell receptors with soluble fluorogenic MHC molecule tetramers which present the antigenic peptide (Altman et al., *Science* 274:94-96, 1996; Dunbar et al., *Curr. Biol.* 8:413-416, 1998). Briefly, soluble MHC class I molecules are folded *in vitro* in the presence of  $\beta$ 2-microglobulin and a peptide antigen which binds the class I molecule. After purification, the MHC/peptide complex is purified and labeled with biotin. Tetramers are formed by mixing the biotinylated peptide-MHC complex with labeled avidin (e.g. phycoerythrin) at a molar ratio of 4:1. Tetramers are then contacted with a source of CTLs such as peripheral blood or lymph node. The tetramers bind CTLs which recognize the peptide antigen/MHC class I complex. Cells bound by the tetramers can be sorted by fluorescence activated cell sorting to isolate the reactive CTLs.

The isolated CTLs then can be expanded *in vitro* for use as described herein. The use of MHC class II molecules as tetramers was recently demonstrated by Crawford et al. (*Immunity* 8:675-682, 1998). Multimeric soluble MHC class II molecules were complexed with a covalently attached peptide. The class II tetramers were shown to bind with appropriate specificity and affinity to specific T cells. Thus tetramers can be used to monitor both CD4<sup>+</sup> and CD8<sup>+</sup> cell responses to vaccination protocols.

The EphA3 HLA binding peptides, as well as complexes of EphA3 HLA binding peptides and HLA molecules, also may be used to produce antibodies, using standard techniques well known to the art. Standard reference works setting forth the general principles of antibody production include Catty, D., Antibodies. A Practical Approach, Vol. 1, IRL Press, Washington DC (1988); Klein, J., Immunology: The Science of Cell-Non-Cell Discrimination, John Wiley and Sons, New York (1982); Kennett, R., et al., Monoclonal Antibodies. Hybridoma, A New Dimension In Biological Analyses, Plenum Press, New York (1980); Campbell, A., Monoclonal Antibody Technology, in Laboratory Techniques and Biochemistry and Molecular Biology, Vol. 13 (Burdon, R. et al. EDS.), Elsevier Amsterdam (1984); and Eisen, H.N., Microbiology, third edition, Davis, B.D. et al. EDS. (Harper & Rowe, Philadelphia (1980).

The antibodies of the present invention thus are prepared by any of a variety of methods, including administering protein, fragments of protein, cells expressing the protein or fragments thereof and an appropriate HLA molecule, and the like to an animal to induce polyclonal antibodies. The production of monoclonal antibodies is according to techniques well known in the art.

Significantly, as is well-known in the art, only a small portion of an antibody molecule, the paratope, is involved in the binding of the antibody to its epitope (see, in general, Clark, W.R. (1986) The Experimental Foundations of Modern Immunology Wiley & Sons, Inc., New York; Roitt, I. (1991) Essential Immunology, 7th Ed., Blackwell Scientific Publications, Oxford). The pFc' and Fc regions, for example, are effectors of the complement cascade but are not involved in antigen binding. An antibody from which the pFc' region has been enzymatically cleaved, or which has been produced without the pFc' region, designated an F(ab')<sub>2</sub> fragment, retains both of the antigen binding sites of an intact antibody. Similarly, an antibody from which the Fc region has been enzymatically cleaved, or which has been produced without the Fc region, designated an Fab fragment, retains one of the antigen

binding sites of an intact antibody molecule. Proceeding further, Fab fragments consist of a covalently bound antibody light chain and a portion of the antibody heavy chain denoted Fd. The Fd fragments are the major determinant of antibody specificity (a single Fd fragment may be associated with up to ten different light chains without altering antibody specificity) and Fd fragments retain epitope-binding ability in isolation.

Within the antigen-binding portion of an antibody, as is well-known in the art, there are complementarity determining regions (CDRs), which directly interact with the epitope of the antigen, and framework regions (FRs), which maintain the tertiary structure of the paratope (see, in general, Clark, 1986; Roitt, 1991). In both the heavy chain Fd fragment and the light chain of IgG immunoglobulins, there are four framework regions (FR1 through FR4) separated respectively by three complementarity determining regions (CDR1 through CDR3). The CDRs, and in particular the CDR3 regions, and more particularly the heavy chain CDR3, are largely responsible for antibody specificity.

It is now well-established in the art that the non-CDR regions of a mammalian antibody may be replaced with similar regions of conspecific or heterospecific antibodies while retaining the epitopic specificity of the original antibody. This is most clearly manifested in the development and use of "humanized" antibodies in which non-human CDRs are covalently joined to human FR and/or Fc/pFc' regions to produce a functional antibody. See, e.g., U.S. patents 4,816,567, 5,225,539, 5,585,089, 5,693,762 and 5,859,205.

Thus, for example, PCT International Publication Number WO 92/04381 teaches the production and use of humanized murine RSV antibodies in which at least a portion of the murine FR regions have been replaced by FR regions of human origin. Such antibodies, including fragments of intact antibodies with antigen-binding ability, are often referred to as "chimeric" antibodies.

Thus, as will be apparent to one of ordinary skill in the art, the present invention also provides for F(ab')<sub>2</sub>, Fab, Fv and Fd fragments; chimeric antibodies in which the Fc and/or FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences; chimeric F(ab')<sub>2</sub> fragment antibodies in which the FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences; chimeric Fab fragment antibodies in which the FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences; and chimeric Fd fragment antibodies in which

the FR and/or CDR1 and/or CDR2 regions have been replaced by homologous human or non-human sequences. The present invention also includes so-called single chain antibodies.

Such antibodies may be used for example to identify tissues expressing protein or to purify protein. Antibodies also may be coupled to specific labeling agents for imaging or to antitumor agents, including, but not limited to, methotrexate, radioiodinated compounds, toxins such as ricin, other cytostatic or cytolytic drugs, and so forth. Antibodies prepared according to the invention also preferably are specific for the peptide/HLA complexes described herein.

Thus, the invention involves polypeptides of numerous size and type that bind specifically to cancer associated antigen polypeptides, and complexes of both cancer associated antigen polypeptides and their binding partners. These polypeptides may be derived also from sources other than antibody technology. For example, such polypeptide binding agents can be provided by degenerate peptide libraries which can be readily prepared in solution, in immobilized form or as phage display libraries. Combinatorial libraries also can be synthesized of peptides containing one or more amino acids. Libraries further can be synthesized of peptoids and non-peptide synthetic moieties.

When "disorder" or "condition" is used herein, it refers to any pathological condition where EphA3, and particularly EphA3 HLA class I or II binding peptides, are expressed. Such disorders include cancers, such as lymphoid tumors, etc.

Some therapeutic approaches based upon the disclosure are premised on inducing a response by a subject's immune system to Eph HLA binding peptide presenting cells. One such approach is the administration of autologous CD4<sup>+</sup> T cells specific to the complex of EphA3 HLA class II binding peptide and an HLA class II molecule to a subject with abnormal cells of the phenotype at issue. It is within the skill of the artisan to develop such CD4<sup>+</sup> T cells *in vitro*. Generally, a sample of cells taken from a subject, such as blood cells, are contacted with a cell presenting the complex and capable of provoking CD4<sup>+</sup> T lymphocytes to proliferate. The target cell can be a transfectant, such as a COS cell, or an antigen presenting cell bearing HLA class II molecules, such as dendritic cells or B cells. These transfectants present the desired complex of their surface and, when combined with a CD4<sup>+</sup> T lymphocyte of interest, stimulate its proliferation. COS cells are widely available, as are other suitable host cells. Specific production of CD4<sup>+</sup> T lymphocytes is described below. The clonally expanded autologous CD4<sup>+</sup> T lymphocytes then are administered to the subject. The

CD4<sup>+</sup> T lymphocytes then stimulate the subject's immune response, thereby achieving the desired therapeutic goal. Autologous CD8<sup>+</sup> T cells responsive to Eph HLA class I binding peptides can be prepared and administered similarly.

The foregoing therapy assumes that at least some of the subject's abnormal cells present the relevant HLA/peptide complex. This can be determined very easily, as the art is very familiar with methods for identifying cells which present a particular HLA molecule, as well as how to identify cells expressing DNA of the pertinent sequences, in this case an EphA3 sequence.

The foregoing therapy is not the only form of therapy that is available in accordance with the invention. CD4<sup>+</sup> T lymphocytes can also be provoked *in vivo*, using a number of approaches. One approach is the use of non-proliferative cells expressing the complex. The cells used in this approach may be those that normally express the complex, such as dendritic cells or cells transfected with one or both of the genes necessary for presentation of the complex. Chen et al., (*Proc. Natl. Acad. Sci. USA* 88: 110-114, 1991) exemplifies this approach, showing the use of transfected cells expressing HPV-E7 peptides in a therapeutic regime. Various cell types may be used. Similarly, vectors carrying one or both of the genes of interest may be used. Viral or bacterial vectors are especially preferred. For example, nucleic acids which encode an EphA3 HLA class II binding peptide may be operably linked to promoter and enhancer sequences which direct expression of the EphA3 HLA class II binding peptide in certain tissues or cell types. The nucleic acid may be incorporated into an expression vector. Expression vectors may be unmodified extrachromosomal nucleic acids, plasmids or viral genomes constructed or modified to enable insertion of exogenous nucleic acids, such as those encoding EphA3 HLA class II binding peptides. Nucleic acids encoding an EphA3 HLA class II binding peptide also may be inserted into a retroviral genome, thereby facilitating integration of the nucleic acid into the genome of the target tissue or cell type. In these systems, the gene of interest is carried by a microorganism, e.g., a *Vaccinia* virus, poxviruses in general, adenovirus, herpes simplex virus, retrovirus or the bacteria BCG, and the materials de facto "infect" host cells. The cells which result present the complex of interest, and are recognized by autologous CD4<sup>+</sup> T cells, which then proliferate.

A similar effect can be achieved by combining a Eph HLA class II binding peptide with an adjuvant to facilitate incorporation into HLA class II presenting cells *in vivo*. If larger than the HLA class II binding portion, the EphA3 HLA class II binding peptide can be

processed if necessary to yield the peptide partner of the HLA molecule while the TRA is presented without the need for further processing. Generally, subjects can receive an intradermal injection of an effective amount of the EphA3 HLA class II binding peptide. Initial doses can be followed by booster doses, following immunization protocols standard in the art.

A preferred method for facilitating incorporation of EphA3 HLA class II binding peptides into HLA class II presenting cells is by expressing in the presenting cells a polypeptide which includes an endosomal targeting signal fused to an EphA3 polypeptide which includes the class II binding peptide. Particularly preferred are EphA3 fusion proteins which contain human invariant chain Ii or LAMP-1. It is also preferred to express separately Ii to enhance presentation of the class II peptide.

Any of the foregoing compositions or protocols can include also Eph HLA class I binding peptides for induction of a cytolytic T lymphocyte response. For example, the EphA3 protein can be processed in a cell to produce both HLA class I and HLA class II responses. By administering EphA3 peptides which bind HLA class I and class II molecules (or nucleic acid encoding such peptides), an improved immune response may be provided by inducing both T helper cells and T killer cells.

In addition, non-EphA3 tumor associated peptides also can be administered to increase immune response via HLA class I and/or class II. It is well established that cancer cells can express more than one tumor associated gene. It is within the scope of routine experimentation for one of ordinary skill in the art to determine whether a particular subject expresses additional tumor associated genes, and then include HLA class I and/or HLA class II binding peptides derived from expression products of such genes in the foregoing EphA3 compositions and vaccines.

Especially preferred are nucleic acids encoding a series of epitopes, known as "polytopes". The epitopes can be arranged in sequential or overlapping fashion (*see, e.g.,* Thomson et al., *Proc. Natl. Acad. Sci. USA* 92:5845-5849, 1995; Gilbert et al., *Nature Biotechnol.* 15:1280-1284, 1997), with or without the natural flanking sequences, and can be separated by unrelated linker sequences if desired. The polytope is processed to generate individual epitopes which are recognized by the immune system for generation of immune responses.

Thus, for example, EphA3 HLA class II binding peptides can be combined with

peptides from other tumor rejection antigens (e.g. by preparation of hybrid nucleic acids or polypeptides) and with EphA3 HLA class I binding peptides to form "polytopes". Exemplary tumor associated peptide antigens that can be administered to induce or enhance an immune response are derived from tumor associated genes and encoded proteins including MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A5, MAGE-A6, MAGE-A7, MAGE-A8, MAGE-A9, MAGE-A10, MAGE-A11, MAGE-A12, GAGE-1, GAGE-2, GAGE-3, GAGE-4, GAGE-5, GAGE-6, GAGE-7, GAGE-8, GAGE-9, BAGE-1, RAGE-1, LB33/MUM-1, PRAME, NAG, MAGE-Xp2 (MAGE-B2), MAGE-Xp3 (MAGE-B3), MAGE-Xp4 (MAGE-B4), tyrosinase, brain glycogen phosphorylase, Melan-A, MAGE-C1, MAGE-C2, MAGE-C3, MAGE-C4, MAGE-C5, NY-ESO-1, LAGE-1, SSX-1, SSX-2 (HOM-MEL-40), SSX-4, SSX-5, SCP-1 and CT-7. For example, antigenic peptides characteristic of tumors include those listed in Table I below.

**Table I: Exemplary Antigens**

Gene	MHC	Peptide	Position	SEQ ID NO:
MAGE-A1	HLA-A1	EADPTGHSY	161-169	8
	HLA-Cw16	SAYGEPRKL	230-238	9
MAGE-A3	HLA-A1	EVDPIGHLV	168-176	10
	HLA-A2	FLWGPRLV	271-279	11
	HLA-B44	MEVDPIGHLV	167-176	12
BAGE	HLA-Cw16	AARAVFLAL	2-10	13
GAGE-1,2	HLA-Cw16	YRPRPRRY	9-16	14
RAGE	HLA-B7	SPSSNRIRNT	11-20	15
GnT-V	HLA-A2	VLPDVFIRC(V)	2-10/11	16, 17
MUM-1	HLA-B44	EEKLIVVLF	exon 2/intron	18
		EEKLSVVLF (wild type)		19

CDK4	HLA-A2	ACDPHSGHFV	23-32	20
		ARDPHSGHFV (wild type)		21
$\beta$ -catenin	HLA-A24	SYLDSGIHF	29-37	22
		SYLDSGIHS (wild type)		23
Tyrosinase	HLA-A2	MLLAVLYCL	1-9	24
	HLA-A2	YMNGTMSQV	369-377	25
	HLA-A2	YMDGTMSQV	369-377	41
	HLA-A24	AFLPWHRLF	206-214	26
	HLA-B44	SEIWRDIDF	192-200	27
	HLA-B44	YEIWRDIDF	192-200	28
	HLA-DR4	QNILLSNAPLGPQFP	56-70	29
	HLA-DR4	DYSYLQSDPDSFQD	448-462	30
Melan-A <sup>MART-1</sup>	HLA-A2	(E)AAGIGILTV	26/27-35	31, 32
	HLA-A2	ILTVILGVL	32-40	33
gp100 <sup>Pmel117</sup>	HLA-A2	KTWGQYWQV	154-162	34
	HLA-A2	ITDQVPFSV	209-217	35
	HLA-A2	YLEPGPVTA	280-288	36
	HLA-A2	LLDGTATLRL	457-466	37
	HLA-A2	VLYRYGSFSV	476-485	38
PRAME	HLA-A24	LYVDSLFFL	301-309	39
MAGE-A6	HLA-Cw16	KISGGPRISYPL	292-303	40
NY-ESO-1	HLA-A2	SLLMWITQCFL	157-167	42

HLA-A2	SLLMWITQC	157-165	43
HLA-A2	QLSLLMWIT	155-163	44

Other examples of HLA class I and HLA class II binding peptides will be known to one of ordinary skill in the art (for example, see Coulie, *Stem Cells* 13:393-403, 1995), and can be used in the invention in a like manner as those disclosed herein. One of ordinary skill in the art can prepare polypeptides comprising one or more EphA3 peptides and one or more of the foregoing tumor rejection peptides, or nucleic acids encoding such polypeptides, according to standard procedures of molecular biology.

Thus polytopes are groups of two or more potentially immunogenic or immune response stimulating peptides which can be joined together in various arrangements (e.g. concatenated, overlapping). The polytope (or nucleic acid encoding the polytope) can be administered in a standard immunization protocol, e.g. to animals, to test the effectiveness of the polytope in stimulating, enhancing and/or provoking an immune response.

The peptides can be joined together directly or via the use of flanking sequences to form polytopes, and the use of polytopes as vaccines is well known in the art (see, e.g., Thomson et al., *Proc. Acad. Natl. Acad. Sci USA* 92(13):5845-5849, 1995; Gilbert et al., *Nature Biotechnol.* 15(12):1280-1284, 1997; Thomson et al., *J. Immunol.* 157(2):822-826, 1996; Tam et al., *J. Exp. Med.* 171(1):299-306, 1990). For example, Tam showed that polytopes consisting of both MHC class I and class II binding epitopes successfully generated antibody and protective immunity in a mouse model. Tam also demonstrated that polytopes comprising "strings" of epitopes are processed to yield individual epitopes which are presented by MHC molecules and recognized by CTLs. Thus polytopes containing various numbers and combinations of epitopes can be prepared and tested for recognition by CTLs and for efficacy in increasing an immune response.

It is known that tumors express a set of tumor antigens, of which only certain subsets may be expressed in the tumor of any given patient. Polytopes can be prepared which correspond to the different combination of epitopes representing the subset of tumor rejection antigens expressed in a particular patient. Polytopes also can be prepared to reflect a broader spectrum of tumor rejection antigens known to be expressed by a tumor type. Polytopes can be introduced to a patient in need of such treatment as polypeptide structures, or via the use of

nucleic acid delivery systems known in the art (see, e.g., Allsopp et al., *Eur. J. Immunol.* 26(8):1951-1959, 1996). Adenovirus, pox virus, Ty-virus like particles, adeno-associated virus, plasmids, bacteria, etc. can be used in such delivery. One can test the polytope delivery systems in mouse models to determine efficacy of the delivery system. The systems also can be tested in human clinical trials.

As part of the immunization protocols, substances which potentiate the immune response may be administered with nucleic acid or peptide components of a cancer vaccine. Such immune response potentiating compound may be classified as either adjuvants or cytokines. Adjuvants may enhance the immunological response by providing a reservoir of antigen (extracellularly or within macrophages), activating macrophages and stimulating specific sets of lymphocytes. Adjuvants of many kinds are well known in the art; specific examples include MPL (SmithKline Beecham), a congener obtained after purification and acid hydrolysis of *Salmonella minnesota* Re 595 lipopolysaccharide, QS21 (SmithKline Beecham), a pure QA-21 saponin purified from *Quillja saponaria* extract, DQS21, described in PCT application WO96/33739 (SmithKline Beecham), vitamin E and various water-in-oil emulsions prepared from biodegradable oils such as squalene and/or tocopherol. Cytokines are also useful in vaccination protocols as a result of lymphocyte stimulatory properties. Many cytokines useful for such purposes will be known to one of ordinary skill in the art, including interleukin-12 (IL-12) which has been shown to enhance the protective effects of vaccines (*Science* 268: 1432-1434, 1995), GM-CSF and IL-18.

There are a number of additional immune response potentiating compounds that can be used in vaccination protocols. These include costimulatory molecules provided in either protein or nucleic acid form. Such costimulatory molecules include the B7-1 and B7-2 (CD80 and CD86 respectively) molecules which are expressed on dendritic cells (DC) and interact with the CD28 molecule expressed on the T cell. This interaction provides costimulation (signal 2) to an antigen/MHC/TCR stimulated (signal 1) T cell, increasing T cell proliferation, and effector function. B7 also interacts with CTLA4 (CD152) on T cells and studies involving CTLA4 and B7 ligands indicate that the B7-CTLA4 interaction can enhance antitumor immunity and CTL proliferation (Zheng et al., *Proc. Nat'l Acad. Sci. USA* 95:6284-6289, 1998).

B7 typically is not expressed on tumor cells so they are not efficient antigen presenting cells (APCs) for T cells. Induction of B7 expression would enable the tumor cells to stimulate

more efficiently CTL proliferation and effector function. A combination of B7/IL-6/IL-12 costimulation has been shown to induce IFN-gamma and a Th1 cytokine profile in the T cell population leading to further enhanced T cell activity (Gajewski et al., *J. Immunol.* 154:5637-5648, 1995). Tumor cell transfection with B7 has been discussed in relation to *in vitro* CTL expansion for adoptive transfer immunotherapy by Wang et al. (*J. Immunother.* 19:1-8, 1996). Other delivery mechanisms for the B7 molecule would include nucleic acid (naked DNA) immunization (Kim et al., *Nature Biotechnol.* 15:7:641-646, 1997) and recombinant viruses such as adeno and pox (Wendtner et al., *Gene Ther.* 4:726-735, 1997). These systems are all amenable to the construction and use of expression cassettes for the coexpression of B7 with other molecules of choice such as the antigens or fragment(s) of antigens discussed herein (including polytopes) or cytokines. These delivery systems can be used for induction of the appropriate molecules *in vitro* and for *in vivo* vaccination situations. The use of anti-CD28 antibodies to directly stimulate T cells *in vitro* and *in vivo* could also be considered. Similarly, the inducible co-stimulatory molecule ICOS which induces T cell responses to foreign antigen could be modulated, for example, by use of anti-ICOS antibodies (Hutloff et al., *Nature* 397:263-266, 1999).

Lymphocyte function associated antigen-3 (LFA-3) is expressed on APCs and some tumor cells and interacts with CD2 expressed on T cells. This interaction induces T cell IL-2 and IFN-gamma production and can thus complement but not substitute, the B7/CD28 costimulatory interaction (Parra et al., *J. Immunol.*, 158:637-642, 1997; Fenton et al., *J. Immunother.*, 21:95-108, 1998).

Lymphocyte function associated antigen-1 (LFA-1) is expressed on leukocytes and interacts with ICAM-1 expressed on APCs and some tumor cells. This interaction induces T cell IL-2 and IFN-gamma production and can thus complement but not substitute, the B7/CD28 costimulatory interaction (Fenton et al., 1998). LFA-1 is thus a further example of a costimulatory molecule that could be provided in a vaccination protocol in the various ways discussed above for B7.

Complete CTL activation and effector function requires Th cell help through the interaction between the Th cell CD40L (CD40 ligand) molecule and the CD40 molecule expressed by DCs (Ridge et al., *Nature* 393:474, 1998; Bennett et al., *Nature* 393:478, 1998; Schoenberger et al., *Nature* 393:480, 1998). This mechanism of this costimulatory signal is likely to involve upregulation of B7 and associated IL-6/IL-12 production by the DC (APC).

The CD40-CD40L interaction thus complements the signal 1 (antigen/MHC-TCR) and signal 2 (B7-CD28) interactions.

The use of anti-CD40 antibodies to stimulate DC cells directly, would be expected to enhance a response to tumor associated antigens which are normally encountered outside of an inflammatory context or are presented by non-professional APCs (tumor cells). Other methods for inducing maturation of dendritic cells, e.g., by increasing CD40-CD40L interaction, or by contacting DCs with CpG-containing oligodeoxynucleotides or stimulatory sugar moieties from extracellular matrix, are known in the art. In these situations Th help and B7 costimulation signals are not provided. This mechanism might be used in the context of antigen pulsed DC based therapies or in situations where Th epitopes have not been defined within known tumor associated antigen precursors.

When administered, the therapeutic compositions of the present invention are administered in pharmaceutically acceptable preparations. Such preparations may routinely contain pharmaceutically acceptable concentrations of salt, buffering agents, preservatives, compatible carriers, supplementary immune potentiating agents such as adjuvants and cytokines and optionally other therapeutic agents.

The preparations of the invention are administered in effective amounts. An effective amount is that amount of a pharmaceutical preparation that alone, or together with further doses, stimulates the desired response. In the case of treating cancer, the desired response is inhibiting the progression of the cancer. This may involve only slowing the progression of the disease temporarily, although more preferably, it involves halting the progression of the disease permanently. In the case of inducing an immune response, the desired response is an increase in antibodies or T lymphocytes which are specific for the EphA3 immunogen(s) employed. These desired responses can be monitored by routine methods or can be monitored according to diagnostic methods of the invention discussed herein.

Where it is desired to stimulate an immune response using a therapeutic composition of the invention, this may involve the stimulation of a humoral antibody response resulting in an increase in antibody titer in serum, a clonal expansion of cytotoxic lymphocytes, or some other desirable immunologic response. It is believed that doses of immunogens ranging from one nanogram/kilogram to 100 milligrams/kilogram, depending upon the mode of administration, would be effective. The preferred range is believed to be between 500 nanograms and 500 micrograms per kilogram. The absolute amount will depend upon a

variety of factors, including the material selected for administration, whether the administration is in single or multiple doses, and individual patient parameters including age, physical condition, size, weight, and the stage of the disease. These factors are well known to those of ordinary skill in the art and can be addressed with no more than routine experimentation.

### Examples

A gene coding for an antigen presented by HLA-DR molecules to anti-melanoma T cells has been identified. T cell clone 19 recognizes a peptide encoded by gene EphA3 and presented by HLA-DRB1\*1101 molecules.

#### **Example 1: Derivation of T cell clone 19**

Blood mononuclear cells ( $2 \times 10^6$  cells/2 ml) collected from melanoma patient LB33 in 1990 were stimulated by the addition of irradiated (100 Gray from a  $^{137}$ Cesium source) autologous melanoma cells LB33-MEL.A-1.DQ.40, referred to as MEL.AQ. These cells were derived from LB33-MEL.A-1 cells (Lehmann et al., *Eur. J. Immunol.* 25:340-347, 1995) by transfection with constructs coding for HLA-DQ $\alpha$  and DQ $\beta$  chains. As a result, MEL.AQ cells carry HLA-DQ molecules, whereas the parental MEL.A-1 cells do not express the HLA-DQ genes.

Tumor cells were systematically treated with IFN $\gamma$  over 48 h before being used as stimulator cells. The incubation of lymphocytes and tumor cells was carried out in Iscove's Dulbecco medium supplemented with L-arginine (116 mg/L), L-asparagine (36 mg/L), L-glutamine (216 mg/L),  $5 \times 10^{-5}$  M 2-mercaptoethanol (AAGM), 10% human serum (HS: a pool of serum from blood donors) (the foregoing is referred to as "medium"), recombinant human IL-2 (20 U/ml) and recombinant human IL-4 (5 U/ml).

On day 7 the responding lymphocytes ( $1.5 \times 10^6$ ) were collected and restimulated as on day 0. On day 10, the lymphocytes ( $3.7 \times 10^6$ ) were collected and CD4 $^{+}$  T cells were sorted as follows. The lymphocytes were labeled with antibodies recognizing B lymphocytes, NK cells, monocytes and CD8 $^{+}$  T cells, and coupled to magnetic microbeads (MACS CD4 $^{+}$  T cell isolation kit, Miltenyi Biotech, Germany). The labeled cells were retained by passage through a magnetic column. The unlabeled CD4 cells ( $3 \times 10^5$ ) were recovered and incubated in medium with IL-2 and IL-4. On day 14, the lymphocytes ( $2 \times 10^6$ ) were restimulated with

tumor cells and cytokines as described previously. On day 21, the lymphocytes were cloned by limiting dilution and stimulated by the addition of irradiated tumor cells ( $10^4$  cells/microwell), irradiated allogenic EBV-transformed B cells LG2-EBV ( $10^5$  cells/well), and medium with IL-2 (20 U/ml) and IL-4 (5 U/ml). The microcultures were restimulated each week with tumor cells, feeder cells and cytokines. One of the CD4 T cell clones derived from this experiment is clone LB33-CTL-443A/19, referred to as T cell clone 19.

### Example 2: Characterization of T cell clone 19

The specificity of clone 19 was analyzed by measuring the production of cytokines during incubation with tumor cells. The cytokine assay uses the M-07e cells, which proliferate in the presence of any of several growth factors, including GM-CSF, IL-3, IL-6 and IL-15 (Avanzi et al., *J. Cell. Physiol.* 145:458-464, 1990; Meazza et al., *Int. J. Cancer* 78:189-195, 1998). It is not known which one or more of these cytokines is produced by clone 19. M-07e cells were kept in Iscove's medium supplemented with AAG, 10% FCS and 50 ng/ml of rhIL-3 (Sandoz).

Briefly, clone 19 (5000 cells/well) was incubated in microwells (100  $\mu$ l) with the indicated stimulator cells ( $3 \times 10^4$  cells/well) in medium containing IL-2 (25 U/ml). After 24 hours, 50  $\mu$ l of medium was collected and added to M-07e cells ( $10^4$  cells/microwell). After 24 hours of incubation,  $^3$ H-thymidine was added and after another 16 hours the cells were harvested for the measurement of thymidine incorporation. As shown in Table 1, clone 19 recognized autologous melanoma cells, but did not recognize autologous EBV-transformed B lymphocytes.

Table 1. Stimulation of clone 19 with autologous tumor cells

Stimulator cells	Proliferation of M-07e cells (cpm)
-	321
MEL.AQ	39258
LB33-EBV	3004

For the analysis of cell surface expression of HLA class II molecules tumor cells were incubated with monoclonal antibodies Leu-10 (anti-HLA-DQ), L243 (anti-HLA-DR) and B7/21 (anti-HLA-DP) from Becton Dickenson for 30 min at 4°C in 134nM NaCl, 5 mM KCl,

0.4 mM  $\text{MSO}_4$ , 0.3mM  $\text{MgCl}_2$ , 5 mM glucose, 4 mM  $\text{NaHCO}_3$ , 1 mM EDTA, penicillin (500 U/ml), streptomycin (1  $\mu\text{g/ml}$ ) and 1% FCS, and buffered with phosphate (1 mM, pH 7.4). the cells were washed, incubated further with FITC-conjugated anti-mouse Ig for 20 min at 4°C, fixed with 0.5% paraformaldehyde and analyzed by flow cytometry.

The MEL.A-1 cells were incubated over two days in medium alone or supplemented with IFN- $\gamma$ , and labeled with monoclonal antibodies specific for HLA-DP, DQ or DR molecules. In the absence of IFN- $\gamma$  about 15% of the cells expressed HLA-DR, but not HLA-DP or HLA-DQ molecules, whereas the other cells were not labeled at all by any of the three antibodies. After incubation with IFN- $\gamma$ , all the cells carried HLA-DP and HLA-DR molecules at levels comparable that found on autologous EBV-transformed B cells. HLA-DQ molecules were not detected on the tumor cells.

To analyze the HLA restriction of clone 19, the lymphocytes were incubated with autologous tumor cells in the presence of an anti-HLA-DR monoclonal antibody (L243; IgG2a anti-HLA-DR, available through the ATTC; 1/30 dilution of ascites fluid containing the antibody). The MEL.A-1.1 cells were derived from MEL.A-1 by selection in vitro for resistance to lysis by CTL clone 159/3 (Lehman et al., 1995). These cells have lost expression of antigen LB33-A, but they are still recognized by all the other anti-LB33-MEL CTL clones, as well as by T cell Clone 19. As indicated in Table 2, the stimulation of the clone was abrogated by the anti-HLA-DR antibody.

Clone 19 recognized the allogenic melanoma cells LB4-MEL derived from patient LB4, indicating that it recognized an antigen that was expressed by at least two melanoma cell lines. Patients LB4 and LB33 are typed HLA-DR11 by serology.

**Table 2. Stimulation of clone 19 with tumor cells.**

Stimulator cells	Proliferation of M-07e cells (cpm)
-	129
MEL.A-1.1 (HLA-DR11)	20172
MEL.A-1.1 + anti-DR	147
LB4-MEL (HLA-DR11)	37534
LB4-MEL + anti-DR	252

The HLA-DR11 serological typing corresponds to the expression of two HLA-DR molecules, sharing a common  $\alpha$  chain and differing by their  $\beta$  chains. The HLA-DR molecules of patient LB33 contain the DRB1\*1101 or DRB3\*0202 chain.

To identify the HLA-DR molecule presenting the antigen recognized by clone 19, allogenic melanoma cell lines derived from DR11 negative patients (MZ2-MEL, LB34-MEL, LG2-MEL) were transfected with constructs encoding the two  $\beta$  chains. cDNA clones encoding the HLA-DRB1\*1101 and DRB3\*0202 chains of patient LB33 were obtained as follows. RNA prepared from LB33-EBV-B cells was converted to cDNA with M-MLV reverse transcriptase (Boehringer Mannheim) using an oligo-dT primer according to the manufacturer's instructions. The cDNA was used as a template for a PCR amplification with primers PCX3DR (5'-CGCGGATCCAGCATGGTGTGTCTG; SEQ ID NO:57) and PCX4DR (5'-GGAATTCCTCAGCTAGGAATCCTGTTG; SEQ ID NO:58). The PCR product was purified using the QIAquick PCR purification kit (Qiagen), digested with BamHI and EcoRI, and ligated into expression vector pcDNA3 (Invitrogen). The constructs were transfected by electroporation into *E. coli* DH5 $\alpha$  and plasmid DNA extracted from several independent colonies was sequenced.

Melanoma cells LB34-MEL were recognized by clone 19 after transfection with the DRB1 sequence but not after transfection with the DRB3 construct (Fig. 1). These results indicated that HLA-DR molecules containing the DRB1\*1101 chain presented the antigen recognized by clone 19. The antigen recognized by clone 19 was named LB33-Z.

### Example 3: A genetic approach to identification of antigens presented by MHC class II

After treatment with IFN- $\gamma$ , MEL.A-1 cells expressed the genes encoding the invariant chain (Ii) and the HLA-DMA and DMB chains, which are necessary to process antigens presented by HLA class II molecules. To identify a gene coding for HLA class I presented antigens, a cDNA library prepared from a tumor is cotransfected into 293-EBNA1 cells with a cDNA encoding the appropriate HLA molecule. This approach was modified to identify a gene coding for LB33-Z antigen (presented by HLA class II), because 293-EBNA1 cells do not express the *HLA-DR $\alpha$* , *DR $\beta$* , *DMA*, *DMB* and *Ii* genes which are necessary to process antigens presented by HLA class II. Expression of these genes is controlled by the class II transactivator (CIITA; Steimle et al., *Cell* 75:135-146, 1993).

293-EBNA1 cells were transfected with a CIITA cDNA (see below) and were verified to express the *HLA-DR $\alpha$* , *DR $\beta$* , *DRB1\*1501*, *DMA*, *DMB* and invariant chain *Ii* genes, and carried HLA-DR molecules. Therefore this modified method was used by cotransfecting 293-EBNA1 cells with a cDNA library from MEL.A-1 and with cDNA clones encoding CIITA and the HLA-DRB1\*1101 chain.

To validate the method, a CD4 T cell clone was used that recognizes a MAGE-A3 peptide presented by HLA-DR13 molecules (Chaux et al., *J. Exp. Med.* 189:767-778, 1999). It was shown that cells expressing HLA-DR13 molecules were recognized by this clone, named clone 37, after transfection with an Ii-MAGE-A3 fusion construct (Chaux et al., 1999). Protein MAGE-A3 seems to be localized in the cytosol, and the Ii-MAGE-A3 protein contains the endosomal targeting signal of Ii, which targets MAGE-A3 into the MHC class II compartments (Sanderson et al., *Proc. Nat'l. Acad. Sci. USA* 92:7217-7221, 1995; Nakano et al., *Science* 275:678-683, 1997).

Various concentrations of the Ii-MAGE-A3 cDNA were cotransfected into 293-EBNA1 cells with the CIITA and DRB1\*1302 cDNA clones. Specifically, 293-EBNA1 cells (50,000 cells/microwell) were cotransfected using lipofectAMINE (Gibco/BRL) with (i) a total amount of 100 ng of expression vector pCEP4, consisting of the indicated amounts of pCEP4-Ii-MAGE-A3 mixed with empty vector, (ii) 10 ng of vector pcDNA3 containing the DRB13 cDNA, (iii) 20 ng of vector EBO76PL containing the CIITA cDNA, with or without 10 ng of vector pcDNA1/Amp containing the Ii cDNA. After 24 h, the anti-MAGE-A3 CD4 clone 37 was added (3000 cells/well). Culture medium was collected after another 16 h and added to M-07e cells for the GM-CSF bioassay. As expected, these transfectants were recognized by clone 37 (Fig. 2). No recognition was observed without cotransfection of the CIITA cDNA.

The efficiency of antigen presentation in this system was attempted to be increased by modifying the amounts of transfected DR $\beta$  and CIITA cDNA, and by adding DR $\alpha$  or Ii cDNA in the cotransfection. Surprisingly, the addition of a cDNA encoding a full-length Ii significantly improved antigen presentation, suggesting that only a limited amount of Ii is obtained through the CIITA-activated transcription. When the 293-EBNA1 cells were cotransfected with cDNA encoding DRB13, CIITA and Ii, and with 200 ng mixtures of pCEP4 and pCEP4 containing the Ii-MAGE-A3 cDNA, a clear recognition of the transfectants by the CD4 clone was obtained with only 0.1 ng of pCEP4-Ii-MAGE-A3.

**Example 4: Identification of cDNA clones encoding antigen LB33-Z**

Total RNA was extracted from MEL.A1 cells by the guanidine-isothiocyanate procedure. Poly(A<sup>+</sup>) RNA enriched with an oligo(dT)-cellulose column (Pharmacia Biotech, Piscataway, NJ) was converted to cDNA with the Superscript Choice System (Gibco BRL, Gaithersburg, MD) using an oligo (dT) primer containing a Not I site at its 5' end (5'-ATAAGAATGCGGCCGCTAAACTA(T)<sub>18</sub>VZ-3'; SEQ ID NO:1 where V = G, A or C; Z = G, A, T, or C). The cDNA was ligated to Hind III-EcoR I adaptors (Stratagene, Heidelberg, Germany), phosphorylated, digested with Not I and inserted at the Hind III and Not I sites of expression vector pCEP4 (Invitrogen, San Diego, CA). This plasmid contains the EBV origin of replication, resulting in episomal multiplication of the transfected plasmids in the human embryonic kidney cells 293 transfected with the EBV EBNA-1 gene.

*E. coli* DH5 $\alpha$  were transformed by electroporation with the recombinant plasmid and selected with ampicillin (50  $\mu$ g/ml). The library was divided into 528 pools of about 100 cDNA clones. Each pool was amplified for 4 hours and plasmid DNA was extracted using the QIAprep 8 plasmid Kit (Qiagen, Hilden, Germany). Duplicate microcultures of 293-EBNA cells (Invitrogen, San Diego, CA), plated in flat-bottom 96 microwells (3.5 x 10<sup>4</sup>/well) 24 hours before transfection, were cotransfected with 1.5  $\mu$ l of lipofectAMINE reagent (Gibco BRL), 100 ng of plasmid DNA of each pool of the cDNA library, 12 ng of plasmid pcDNA3 (Invitrogen) containing the HLA-DRB1\*1101 cDNA isolated from MEL.A1 cells, 12 ng of plasmid pcDNAI-Amp (Invitrogen) containing a cDNA coding for the Invariant chain (Ii), and 24 ng of plasmid EBO-76pl containing a cDNA coding for the Class II transactivator (CIITA; Steimle et al., *Cell* 75:135-146, 1993) (kindly provided by B. Mach - Department of Genetics and Microbiology, University of Geneva Medical School, Switzerland). After 24 hours clone 19 (5000 cells/well) was added to each microculture of transfected 293-EBNA cells, in 100  $\mu$ l of Iscove's Dulbecco medium supplemented with AAGM, 1% human plasma, and IL-2 (25 U/ml). After another 24 hours 75  $\mu$ l of supernatant was collected and cytokine production was measured with the M-07e cells as described above.

Four pools of cDNA proved positive; two of them were subcloned, and two cDNA clones of 6 Kb (cDNA clone 279) and 2.5 Kb (cDNA clone 60) were found to transfer expression of antigen LB33-Z into 293-EBNA cells cotransfected with cDNA clones encoding HLA-DRB1\*1101, CIITA and Ii (Fig. 3).

**Example 5: Characterization of cDNA clones 60 and 279**

cDNA clones were sequenced using the dideoxy chain method in a Perkin Elmer AB310 automated DNA sequencer. The sequences corresponded to that of the human tyrosine kinase receptor HEK (Accession number M83941; SEQ ID NOs:2 and 3 for nucleic acid and polypeptide, respectively), a member of the Eph family of receptors originally cloned from the human lymphoid tumor cell line LK63 (Wicks et al, *Proc. Natl. Acad. Sci. USA* 89:1611-1615, 1992). According to the Eph Nomenclature Committee, this receptor is now designated EphA3 (Eph Nomenclature Committee, *Cell* 90: 403-404, 1997 (Anderson et al.)).

cDNA 279 is about 6000 bp long (3300 nt of which are set forth as SEQ ID NO:4) and contains a polyA tail and a polyadenylation signal. It contains an ORF of about 2949 nucleotides encoding a protein of about 983 amino acids (SEQ ID NO: 5), corresponding to the complete ORF of the published EphA3 sequence. The coding sequence of cDNA clone 279 is almost identical to that of M83941. They differ by four nucleotide positions resulting in two differences at the amino acid level (Table 3).

**Table 3. Differences between the coding sequences of clone 279 and the published EphA3 cDNA (M83941).**

M83941			DNA clone 279		
nt	codon	aa	nt	codon	aa
<u>2059</u>	<u>AAA</u>	K	<u>2184</u>	<u>AAG</u>	K
<u>2831</u>	<u>TCT</u>	S	<u>2956</u>	<u>ACT</u>	T
<u>2870</u>	<u>Cgg</u>	R	<u>2995</u>	<u>Tgg</u>	W
<u>2902</u>	<u>ggC</u>	G	<u>3027</u>	<u>ggT</u>	G

cDNA 60 is about 2546 bp long (SEQ ID NO:6) and contains an ORF of about 1617 nt encoding a protein of about 539 amino acids (SEQ ID NO:7) corresponding to nt 101-1694 of the reported EphA3 sequence (M83941). Nucleotide 1694 in M83941 presumably corresponds to the end of exon 7, as deduced from a comparison with the sequence of the chicken EphB2 receptor (previously named CEK5; Accession number M62325; Connor et al.,

*Oncogene* 11:2429-2438, 1995). In cDNA clone 60, the reading frame of exon 7 extends for another 23 bp into a putative intronic sequence. As a result, cDNA 60 encodes a protein corresponding to the extracellular portion of EphA3. Such truncated forms corresponding to the extracellular domains of the EphA3 and EphB2 receptors have been described. They appear to be generated through alternative polyadenylation (Sajjadi et al., *New. Biol.* 3:769-778, 1991; Connor et al., 1995; Tang et al., *Oncogene* 17:521-526, 1998).

Fig. 4 is a schematic representation of cDNA clones encoding EphA3, including the M83941 sequence, the clone 60 cDNA sequence, and the clone 279 cDNA sequence. Shaded areas represent the open reading frames.

#### Example 6: Identification of an EphA3 antigenic peptide

293-EBNA1 cells were transfected with constructs coding for membrane-bound truncated proteins corresponding to the extracellular portion of EphA3. Briefly, a sequence coding for the signal sequence of EphA3 was first cloned into vector pcDNA3. Next, cDNA clone 279 was used as a template for a PCR amplification with primers OPC894 (5'-CGCGGATCCCTTCTCCAGCAATCAGAGCGC; SEQ ID NO:45) and OPC895 CCGGAATTCTGAATCCAGTAGATTGACTTCATTGGA; SEQ ID NO:46) in the following conditions: 5 min at 94° C, followed by 30 cycles consisting of 1 min at 94° C, 2 min at 64° C, 3 min at 72° C. The PCR product was purified using QIAquick PCR purification kit (Qiagen), digested with BamH1 and EcoR1 and ligated into pcDNA3, to obtain pcDNA3-EphA3-signal. Subsequently fragments of cDNA clone 279, corresponding to truncated portions of the extracellular part of the receptor, were amplified by PCR using three different sense primers, OPC 899 (5'-CCGGAATTCAAAACAATTCAAGGGGAGCTGGG; SEQ ID NO:47), OPC 941 (5'-CCGGAATTCTGTACCCGACCTCCATCTTCA; SEQ ID NO:48) or OPC 896 (5'-CCGGAATTCTGTGAGCCATGCAGCCCAAATG; SEQ ID NO:49) with the same antisense primer OPC897 (5'-ATAGTTTAGCGGCCGCTCACTTATAGCCACAGAACCTCCCA; SEQ ID NO:50). The three PCR products were cloned into the EcoR1 and Not1 sites of pcDNA3-EphA3-signal. The resulting constructs coded for putative membrane-bound EphA3 receptors truncated at their amino termini and differing from wild-type EphA3 by the insertion of two amino acids (E-F) after the first 10 residues of the mature protein.

Three of these constructs are shown in Fig. 5. They were cotransfected into 293-EBNA1 cells with CIITA, Ii and DRB1\*1101 cDNA clones, and the transfectants were tested for the expression of antigen LB33-Z with CD4 clone 19. The results indicated that the peptide coding region corresponded to nucleotides 1064-1237 of cDNA 279.

5 This region contained two sequences coding for a peptide containing the HLA DR1\*1101 binding motif, namely W or Y or F at position 1, R or K or H at position 6, and A or G or S or P at position 9 (Rammensee et al., *Immunogenetics* 41:178-228, 1995). Two 16-mer peptides containing these motifs were synthesized and incubated with LB33-EBV B cells. CD4 clone 19 was added, and peptide DVTFNIICKKCGWNIK (SEQ ID NO:51; the EphA3 nucleotide sequence that encodes the first 15 amino acids of this peptide, SEQ ID NO:52, is shown in Fig. 5) sensitized cells to recognition with a half-maximal effect at 1  $\mu$ M (Fig. 6A). The shortest recognized peptide was the core nonamer (FNIICKKCG; SEQ ID NO:53) containing the DR11 binding motif. Increasing its length by 3 residues at the amino terminus (DVTFNIICKKCG; SEQ ID NO:54 improved recognition by a factor of 3. Longer peptides, with additional residues at the carboxy terminus, were not recognized better. Peptides lacking even one of the core nonamer residues (NIICKKCGWNIKQCEP; SEQ ID NO:59 and DVTFNIICKKC; SEQ ID NO:60) were not well recognized (Fig. 6A).

The foregoing illustrate and represent a simple genetic approach that is generally applicable to clone genes encoding antigens presented by MHC class II molecules. It includes  
20 cloning a cDNA library in an expression plasmid containing the EBV origin of replication, and cotransfecting the library into 293-EBNA1 cells or similar cells together with cDNA clones coding for CIITA and for the relevant HLA class II chains.

There are two differences between this protocol and that used for the identification of mutated melanoma antigens by Wang and coworkers (*Science* 284:1351-1354, 1999; *J. Exp.*  
25 *Med.* 189:757-765, 1999). First, the cDNA library was not cloned in-frame with an endosomal targeting sequence, such as that of Ii or LAMP. The antigenic protein is naturally processed through the class II pathway in the tumor cells, and thus it is believed that the full-length and properly folded protein reached the MHC compartment by itself in the 293-EBNA1 transfectants. In the course of experiments to identify the antigenic peptide (as  
30 described above), it was observed that targeting EphA3 into the endosomes was less efficient in terms of antigenicity. This result was wholly unexpected and surprising. Constructs corresponding to the extracellular portion of the receptor (EphA3-EC) or the EphA3-EC in

fusion with the 80 carboxy terminal residues of Ii were transfected into 293-EBNA1 cells. Transfectants expressing the Ii-EphA3-EC product were recognized 20 times less efficiently than those expressing EphA3-EC. Therefore if the cDNA library had been cloned in frame with Ii(1-80), most probably the EphA3 cDNA clones would not have been detected as they were diluted in the cDNA pools.

Second, the library was cotransfected with cDNA clones encoding CIITA and DR $\beta$ , instead of cDNA clones coding for DR $\alpha$ , DR $\beta$ , Ii, DMA and DMB. Even though transfection with CIITA clearly induced the expression of Ii, as tested with RT-PCR, and conferred to 293-EBNA1 cells the capacity to present antigens on HLA class II molecules, it was observed that the additional cotransfection of an Ii cDNA improved antigen presentation by the transfectants. This proved true for antigens encoded by the Ii-MAGE-A3 and EphA3 cDNA clones. This may result from a larger amount of Ii protein or from the fact that Ii is present earlier after transfection of an Ii cDNA than after transfection of the CIITA cDNA.

#### **Example 7: Modification of the EphA3 antigenic peptide**

The EphA3 antigenic peptide contains two cysteine residues, at positions 5 and 8 of the core peptide (SEQ ID NO:53). Considering that modifications of cysteine residues were shown to strongly influence the recognition of several antigenic peptides, variants of SEQ ID NO:54 were prepared by mutating one or both of the cysteine residues. As shown in Fig. 6B, normal (SEQ ID NO:54) and variant (SEQ ID NO:61, 62 and 63) EphA3 peptides were tested for recognition by clone 19 as described above. Only SEQ ID NO:54 and SEQ ID NO:62 were efficiently recognized. Therefore, the cysteine residue at position 8 of SEQ ID NO:54 appears to be required for efficient recognition by T cell clone 19. In contrast, the cysteine residue at position 11 of SEQ ID NO:54 is not required for recognition; the mutant peptide SEQ ID NO:62 is recognized slightly more efficiently than the normal peptide. Other modifications are made to the amino acid sequence of the EphA3 peptides in a similar fashion to prepare other functional variants.

#### **Example 8: Expression of the gene EphA3**

Expression of EphA3 in normal tissues was studied by reverse transcription-polymerase chain reaction (RT-PCR) amplification (Fig. 7). Total RNA extraction and reverse transcription of RNA were performed as described previously (Van den Eynde et al. *J. Exp.*

Med. 182(3):689-698, 1995). For the analysis of EphA3 expression in tumor and normal tissues samples, PCR primers were OPC 818 (5'-AGCAACATGGATTGTCAGCTCTC; SEQ ID NO:55) and OPC 806 (5'-TGTTGGTGAGTCCAAACTGTCG; SEQ ID NO:56), the position of which is shown in Fig. 4. PCR conditions were 5 min at 94°C, followed by 32 cycles consisting of 1 min at 94°C, 2 min at 65° C, 3 min at 72° C. It was verified that these conditions placed the reactions in the linear range of DNA amplification. The quality of RNA preparations was tested by PCR amplification of a human  $\beta$ -actin sequence. The quantities of the amplified DNA were visually assessed on agarose gels stained with ethidium bromide. Band intensities were compared with that of PCR products of serial dilutions (1:1, 1:3, 1:9, and 1:27) of reverse transcribed RNA from MEL.A-1 cells. The level of expression of each sample was normalized for RNA integrity by taking into account the level of expression of the  $\beta$ -actin gene.

High levels of EphA3 expression were found in foetal brain and retina. Samples of adult brain, colon, liver, bladder and prostate expressed the EphA3 gene at levels between 10 and 30% of that found in MEL.A-1 cells. Samples of skin, muscle, lung, kidney, adrenals, ovary, testis, heart, liver and breast expressed between 3-10% of that level. In several tissues, no expression of EphA3 was detected. It is important to note that these EphA3 negative samples include all the tissues and cells that are expected to carry HLA class II molecules, such as bone marrow, blood mononuclear cells, thymus, EBV transformed B lymphocytes, or CTL clones. These results indicate that although EphA3 is expressed in many tissues, there should be no presentation of EphA3 antigenic peptides by HLA class II molecules on normal cells. This is because even though normal cells may express HLA class II or EphA3 genes, apparently none of them coexpress these genes and present the antigen.

Expression of EphA3 in tumor samples and cell lines was also studied by RT-PCR as described above (Fig. 8). Significant proportions of tumors, such as 44% (11/25) of small cell lung cancer, 24% (10/41) of non small cell lung cancer, 58% (17/29) of sarcomas, or 31% (12/38) of renal cell carcinomas, express EphA3 at a level that corresponds to 10% of that found in MEL.A-1. This level of expression is higher than that found in the corresponding normal tissues.

Melanomas often express EphA3, whereas melanocytes do not. About 20% (10/51) of the melanoma samples expressed EphA3 at a high level, comparable to that of MEL.A-1. No significant difference was observed between samples of primary or metastatic melanomas.

EphA3 was expressed at a high level by 76% (22/29) of the melanoma lines, a proportion significantly higher than that of the positive metastatic samples. For 8 lines, we observed that the level of EphA3 expression in the original tumor sample was at least 30 times lower. This did not result from much lower proportions of tumor cells in the samples, as the levels of expression of the actin and tyrosinase genes were comparable in the samples and corresponding cell lines. These results suggested either that only a fraction of the melanoma cells expressed EphA3 at a high level and that these cells were selected during the establishment of the cell lines, or that EphA3 expression was induced *in vitro*.

#### **Example 9: Identification of additional EphA3 HLA-DR restricted peptides**

To identify the EphA3 peptides recognized by CD4<sup>+</sup> clones, short peptides, corresponding to 16-20 amino acid fragments of the EphA3 protein sequence are synthesized, loaded on autologous EBV-B cells (B cells transformed with Epstein-Barr virus) and tested for recognition by clone 19 T cells. Peptides are synthesized using F-moc for transient NH<sub>2</sub>-terminal protection and are characterized using mass spectrometry. Lyophilized synthetic peptides are dissolved in DMSO (Merck) and used at a final concentration of 500  $\mu$ M, 50  $\mu$ M or 5  $\mu$ g/ml. EBV-B cells (5,000 per round-bottomed microwell) are incubated 2 hours at 37°C, 8% CO<sub>2</sub> in the presence of the different peptides at various. Clone 19 cells are then added at 2,500 cells per well. The assay medium is Iscove's medium supplemented with L-glutamine, L-arginine, L-asparagine, 10% human serum and IL-2 (25 U/ml). After 18-20 hours, supernatants are harvested and assessed for TNF- $\alpha$  and/or IFN- $\gamma$  secretion (e.g., using a standard ELISA test) or by the M-07e cell assay described above.

In one set of experiments, the peptides are screened at a non-physiologic concentration of 500  $\mu$ M. Non-physiologic concentrations of peptide may lead to non-specific activation of T cells clones. Peptides which are effective at 500  $\mu$ M but are not effective in activating T cell clones when used at 50  $\mu$ M are not considered HLA class II binding peptides. Those peptides which stimulate specifically TNF- $\alpha$  and IFN- $\gamma$  production by T cell clones when used at more physiologic concentrations or preferably lower concentrations are considered HLA class II binding peptides.

#### **Example 10: Determination of minimal EphA3 peptides able to stimulate T cells**

Unlike HLA-class I-restricted peptides, class II-restricted peptides vary considerably

in length and can tolerate extensions at both the amino and carboxy termini. Shortened peptides having deletions of one residue or more are prepared and tested for stimulation of clone 19 cells as described in Examples 6 and 9.

#### 5 **Example 11: Preparation and use of EphA3 fusion proteins**

The EphA3 protein can be expressed as a fusion protein with an endosomal targeting polypeptide such as invariant chain (Ii) or lysosome-associated membrane protein (LAMP-1) to target the presentation of EphA3 derived peptides to the HLA class II presentation pathway. The fusion proteins are prepared according to standard molecular biology techniques.

10 Plasmids containing the human invariant chain and LAMP-1 encoding cDNAs have been described (*J. Cell Science* 106:831-846, 1993; *Proc. Natl. Acad. Sci. USA* 92:11671-11675, 1995). Specific examples of the construction and use of Ii and LAMP-1 fusion proteins for targeting of proteins and peptides to the HLA class II pathway can be found in PCT/US98/18601.

15 Expression of EphA3-endosomal targeting signal fusion proteins also results in peptide presentation in HLA class I. This can be determined, for example, by measuring activation of the EphA3 specific CTLs in accordance with standard procedures such as chromium release assays. Connecting an endosomal targeting signal to EphA3 therefore can be used as a vaccine to induce presentation of EphA3-derived peptides in both HLA class I  
20 and class II.

#### **Example 12: Identification of EphA3 HLA class I binding peptides**

In a first method, available CTL clones directed against antigens presented by autologous tumor cells shown to express EphA3 are screened for specificity against COS cells  
25 transfected with EphA3 nucleic acids (e.g. SEQ ID NO:2, 4 or 6) and autologous HLA alleles as described by Brichard et al. (*Eur. J. Immunol.* 26:224-230, 1996). CTL recognition of EphA3 peptides is determined by measuring release of TNF from the cytolytic T lymphocyte or by <sup>51</sup>Cr release assay (Herin et al., *Int. J. Cancer* 39:390-396, 1987). If a CTL clone specifically recognizes a transfected COS cell, shorter fragments of the coding sequences are  
30 prepared and tested by transfecting COS cells to identify the region of the gene that encodes the peptide recognized by the CTL. Fragments of EphA3 are prepared by exonuclease III digestion or other standard molecular biology methods such as PCR. Synthetic peptides are prepared and tested to confirm the exact sequence of the HLA class I antigen.

Alternatively, CTL clones are generated by stimulating the peripheral blood lymphocytes (PBLs) of a patient with autologous normal cells transfected with DNA clones encoding EphA3 polypeptides (e.g. SEQ ID NO:2, 4, or 6) or with irradiated PBLs loaded with synthetic peptides corresponding to the putative proteins and matching the consensus for the appropriate HLA class I molecule to localize the antigenic peptide within the EphA3 clones (see, e.g., van der Bruggen et al., *Eur. J. Immunol.* 24:3038-3043, 1994; Herman et al., *Immunogenetics* 43:377-383, 1996). Localization of one or more antigenic peptides in a protein sequence can be aided by HLA peptide binding predictions made according to established rules for binding potential (e.g., Parker et al, *J. Immunol.* 152:163, 1994; Rammensee et al., *Immunogenetics* 41:178-228, 1995). HLA binding predictions can conveniently be made using an algorithm available via the Internet on the National Institutes of Health World Wide Web site at URL <http://bimas.dcrt.nih.gov>. For example, several predicted HLA binding motifs for the EphA3 based on SEQ ID NO:3 are listed in the table below:

**Table 4: Predicted HLA class I binding motifs in EphA3**

SEQ ID NO:3 position	HLA molecule	Binding score (t <sub>1/2</sub> disassociation)
AA554-562	A_0201	5534
AA589-597	A_0201	1338
AA341	A_0201	568
AA656-664	A68.1	600
AA558-566	A68.1	400
AA149-157	B60	640
AA895-903	B7	360
AA788-796	B_2705	3000
AA565-573	B_2705	3000
AA683-691	B_2705	2000
AA727-735	B_2705	2000

AA168-176	B_2705	2000
AA103-111	B_2705	2000
AA1-9	B_3701	200
AA929-937	B_4403	600
AA459-467	B_5101	880
AA47-55	B_5102	1064
AA675-683	B_5201	500

Alternatively, CTL clones obtained by stimulation of lymphocytes with autologous tumor cells which express EphA3 are screened for specificity against COS cells transfected with EphA3 cDNA and autologous HLA alleles as described by Brichard et al. (*Eur. J. Immunol.* 26:224-230, 1996).

Optionally, shorter fragments of EphA3 cDNAs are generated by PCR. Shorter fragments are used to provoke TNF release or  $^{51}\text{Cr}$  release as above.

### Example 13: Identification of minimal EphA3 HLA binding peptides

Synthetic peptides corresponding to portions of the shortest fragment of EphA3 which provokes TNF or  $^{51}\text{Cr}$  release are prepared. Progressively shorter peptides are synthesized to determine the optimal EphA3 HLA binding peptides for a given HLA molecule.

Synthetic peptides are tested for lysis of HLA expressing cells according to known procedures. For example, if the HLA which presents a peptide of interest is determined to be HLA-A2, then T2 cells can be used. T2 cells are HLA-A2<sup>+</sup> cells which have an antigen-processing defect resulting in an increased capacity to present exogenous peptides. T2 cells are mixed with a synthetic peptide corresponding to the CTL-reactive portion of EphA3. CTL cells are added and lysis is measured after 4 hours to determine which peptides efficiently stimulate the lysis of T2 cells bearing HLA-A2. Other HLA expressing cells are known in the art or can be prepared by transfection with specific HLA clones.

To determine the optimal size of the synthetic peptide, peptides of decreasing size are synthesized based on the sequence of the peptide determined above, by successively removing one amino acid from the amino terminal end or the carboxy terminal end of the peptide. These peptides are tested for the ability to induce cell lysis of appropriate HLA expressing

cells by CTL cells in a dose response assay. Lyophilized peptides are dissolved at 20 mg/ml in DMSO, then diluted to 2 mg/ml in 10mM acetic acid and stored at -80°C. Target cells, e.g. HLA-A2<sup>+</sup> T2 cells, are labeled with <sup>51</sup>Cr, as described above, for 1 hour at 37°C followed by extensive washing to remove unincorporated label. To confirm the necessity of the  
5 interaction of the peptide with the HLA, T2 cells optionally can be pretreated with an anti-HLA-A2 antibody, such as MA2.1 (Wölfel et al., *Eur. J. Immunol.* 24: 759-764, 1994), and then are incubated in 96-well microplates in the presence of various concentrations of peptides for 30 minutes at 37°C. CTLs which recognize the peptide presented by the HLA are then added in an equal volume of medium at an effector:target ratio of 30:1. Chromium-51 release  
10 is measured after 4 hours.

#### Example 14: Recognition of other Eph proteins by T cell clones

HLA class I or class II binding peptides of EphA3 may be present identically (or with minor variations) in the amino acid sequences of other Eph proteins. Homologous peptide  
15 sequences also may be found in other cancer antigens.

To determine if the CD4<sup>+</sup> T cell clone 19 recognizes other Eph peptides, the recombinant proteins, or synthesized peptides corresponding to the homologous region in these proteins, are used to load antigen presenting cells (such as EBV-B cells) to test for recognition by clone 19 according to the assays described above. Homologous (i.e. non-  
20 identical) peptides which are recognized by clone 19 may be regarded as functional variants of the EphA3 peptides described herein.

Other aspects of the invention will be clear to the skilled artisan and need not be repeated here. Each reference cited herein is incorporated by reference in its entirety.

25 The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, it being recognized that various modifications are possible within the scope of the invention.

30 We claim: